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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–d1 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 α_1 subunit, one of four different auxiliary α_2–δ, and one of four β subunits (for a review see Catterall, 2000). Although the α_1 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 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 α_1 subunit's domain I–II linker region masks an endoplasmic reticulum (ER) retention signal (Bichet *et al*., 2000), thus facilitating translocation of

the α_1 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 β_3 subunit on $Ca_v2.2$ (N-type) calcium channel current has been demonstrated in *Xenopus* oocytes (Yasuda *et al*., 2004). Compared with β subunits, the effect of α_2–δ subunits on HVA calcium channel currents is ambiguous. For instance, in the absence of β subunits, α_2–δ subunits reportedly mediate only a negligible effect on current densities of $Ca_v1.2$, $Ca_v2.1$, $Ca_v2.2$ and $Ca_v2.3$ 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 α_1 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 α_1 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 α_2–δ_1 and the four different β subunits on $Ca_v1.2$ (L-type), $Ca_v2.2$ (N-type) and $Ca_v2.3$ (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, α_2–δ subunits appear as important or more important for regulating whole cell conductance compared with β subunits, and that for Ca_v2 channels, β and α_2–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 α_2–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% CO_2) in Dulbecco's minimal essential medium (DMEM) supplemented with fetal bovine serum, penicillin and streptomycin. Cells were transfected with cDNAs encoding rat α_1 ($Ca_v1.2$, $Ca_v2.2$, $Ca_v2.3$) alone, or in combination with β (β_{1b}, β_{2a}, β_3, β_4) and/or α_2-δ_1, 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 $Ca_v1.2$ and $Ca_v2.2$ channels, 7 μg of each calcium channel subunit plus 4 μg of EGFP cDNA was used for transfection. Owing to the robust expression of $Ca_v2.3$ channels, an additional set of experiments was conducted with 1 μg $Ca_v2.3$ 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 $BaCl_2$, 1 $MgCl_2$, 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 $CsCH_3SO_4$, 4 $MgCl_2$, 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 α_1 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:

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

where E_{rev} is the reversal potential, $V_{1/2,\text{act}}$ is the half-activation potential, $G_{\max}$ is the maximum slope conductance and k_{act} is the slope factor. To analyse faithfully the effects of auxiliary subunits on $G_{\max}$ 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 $G_{\max}$ values. As it was essential that these data were included in Fig. 6, $G_{\max}$ 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_{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:

[β_1 (5'-CCCGGGACATGCTGGTCTTC),
β_2 (5'-GGGGCGTAATTTGAGA),
β_3 (5'-CAGCCAGCTGCGCCTGTG),
β_4 (5'-AATGACAGCAGCCCATTAG),
α_2–δ_1 (5'-TGTCCTGTTTCCTTTGTC),
α_2–δ_2 (5'-CCTGCAGGATCTGGTCACTG),
α_2–δ_3 (5'-GCTACTATAGTTAGGTTTCC),
α_2–δ_4 (5'-ACTGTTGTAGTTAGGTTTGGG)]

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:

[(β_1 (5'-AGGAGGCAGCCGAAGGCC),
β_2 (5'-AAGAAGCAGTCACATAAA),
β_3 (5'-ACTTCAGAACCAGCAGCTG),
β_4 (5'-ATTGAAAGACGAAGTCT),
α_2–δ_1 (5'-ACATAACCGGCCAATTTGAA),
α_2–δ_2 (5'-ACCTGACACAGGATGGCCCTGG),
α_2–δ_3 (5'-CACTCTCCCTCAGGCACA),
α_2–δ_4 (5'-CAAGCTCCTCAGCTCGCAG)].

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_{max}. One-way anova with Dunnett's test as a post test was used for all other comparisons. Asterisks indicate statistical significance +/–β, number symbols indicate statistical significance +/–α_2–δ. 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 β_3 subunits, which play a important role in α_1 subunit expression when the α_1 is injected alone (Tareilus *et al*., 1997; Canti *et al*., 2001). It has been suggested that HEK-293 cells may express two α_2–δ_1 variants and one type of calcium channel β 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 α_2–δ or β subunits in tsA-201 cells, whereas positive control mRNA isolated from either rat brain or human retina (for α_2–δ_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.

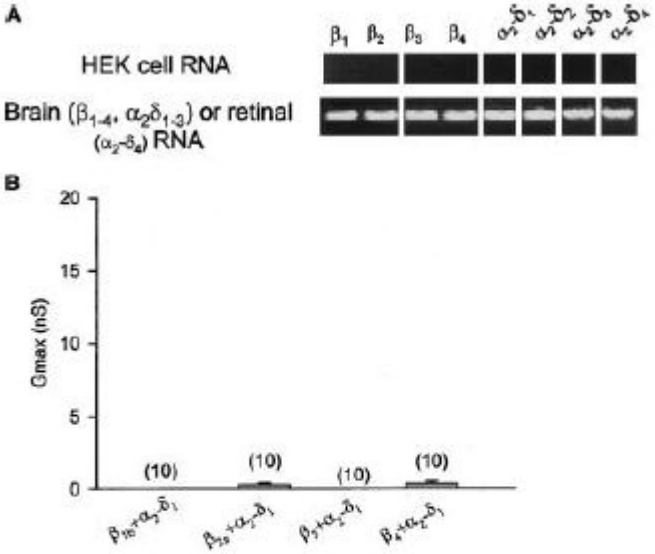

FIG. 1. (A) RT-PCR to detect β_{1-4} and α_2-δ_{1-4} in HEK cell, rat brain and human retina RNA. One microgram of total HEK cell RNA was used in the RT-PCR with oligonucleotides specific to each auxiliary subunit. As a positive control, 1 μg of brain RNA was used to detect β_{1-4} and α_2-δ_{1-3} and 1 μg of retinal RNA was used for the α_2–δ_4 RT-PCR. (B) Whole cell conductance (G_{max}) obtained from cells expressing only auxiliary subunits through fits of macroscopic current voltage relations. Numbers in parentheses reflect numbers of experiments, error bars are standard errors. The scaling on the ordinate was deliberately extended to 20 nS to facilitate comparison with the data shown in Fig. 6.

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 α_1 subunits. We detected no current activity when the channels were coexpressed with a combination of either β_{1b}/α_2–δ_1 or β_3/α_2–δ_1 (Fig.1B, $n = 10$ each). We did, however, detect very small (≈5–10 pA) inward barium currents in some of the cells expressing β_{2a}/α_2–δ_1 or β_4/α_2–δ_1; as we will show below, these were negligible compared with whole cell currents obtained in the presence of any of the α_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, $Ca_v1.2$, $Ca_v2.2$ and $Ca_v2.3$ calcium channel α_1 subunits were transiently transfected into tsA-201 either alone or with various combinations of α_2–δ_1 and β subunits, and resulting depolarization-activated barium currents were characterized using whole cell patch clamp recordings. For $Ca_v2.3$ 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 $Ca_v2.3$ α_1 subunits over any cotransfected auxiliary subunits, an additional set of experiments were carried out in which the amount of $Ca_v2.3$ α_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_2–δ_1, β_{2a} or β_4 subunits with $Ca_v1.2$ channels resulted in a hyperpolarizing shift in half-activation potential that did not occur with Ca_v2 channels. The lack of the effect of β_1 on $Ca_v1.2$ 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_2–δ_1 subunit on $Ca_v1.2$, $Ca_v2.2$ and $Ca_v2.3$ 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_{1b} subunits appeared preferentially to regulate the voltage dependence of activation of $Ca_v2.3$ channels irrespective of the amount of $Ca_v2.3$ α_1 subunit cDNA used for transfection. Surprisingly, β_3 subunits did not cause significant hyperpolarizing shifts for any of the calcium channels tested. Qualitatively similar findings with β_3 subunits have been reported for $Ca_v1.2$ channels expressed in tsA-201 cells (Gerster *et al*., 1999) and for $Ca_v2.2$ 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_1 subunit.

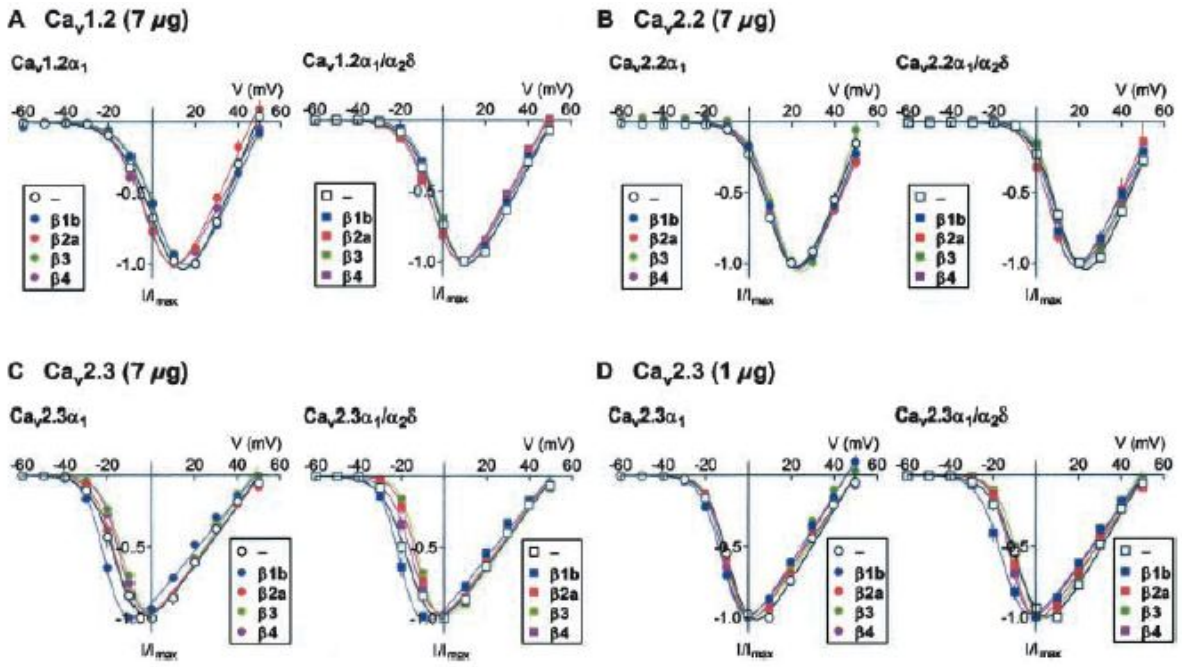

FIG. 2. Effects of subunit coexpression on the current voltage relations of $Ca_v1.2$ (A), $Ca_v2.2$ (B) and $Ca_v2.3$ (C and D) calcium channels. The individual data points are means from 18 to 103 experiments (see Tables 1–3), error bars are in most cases too small to be visible. The *I–V* relations were normalized to overlap at peak to facilitate comparison, and fitted as described in the Materials and methods section. In panels A–C, 7 μg of each cDNA was transfected, in panel D $Ca_v2.3$ cDNA was lowered to 1 μg.

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 not the case. As seen from the raw current records in Fig. 3 and from the ensemble data shown in Fig. 4, coexpression with α_2–δ_1 and/or most of the individual β subunits resulted in a significant change in the inactivation kinetics of Ca_v2 calcium channels. In particular, β_3, which did not affect activation of the channels, mediated a pronounced speeding of $Ca_v2.2$ and $Ca_v2.3$ channel inactivation, in general agreement with previous recordings of $Ca_v2.3$ 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), β_{2a} subunits slowed inactivation of all calcium channel subtypes examined. The β_{1b} subunit preferentially accelerated inactivation kinetics of $Ca_v2.2$ channels. By contrast, β_4 subunits did not appear to be able to regulate inactivation kinetics of the three calcium channel subtypes examined. Unlike with $Ca_v2.2$ and $Ca_v2.3$ channels, the inactivation kinetics of $Ca_v1.2$ channels were, with the exception of β_{2a}, 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 $Ca_v2.2$ channels (Stephens *et al*., 2000).

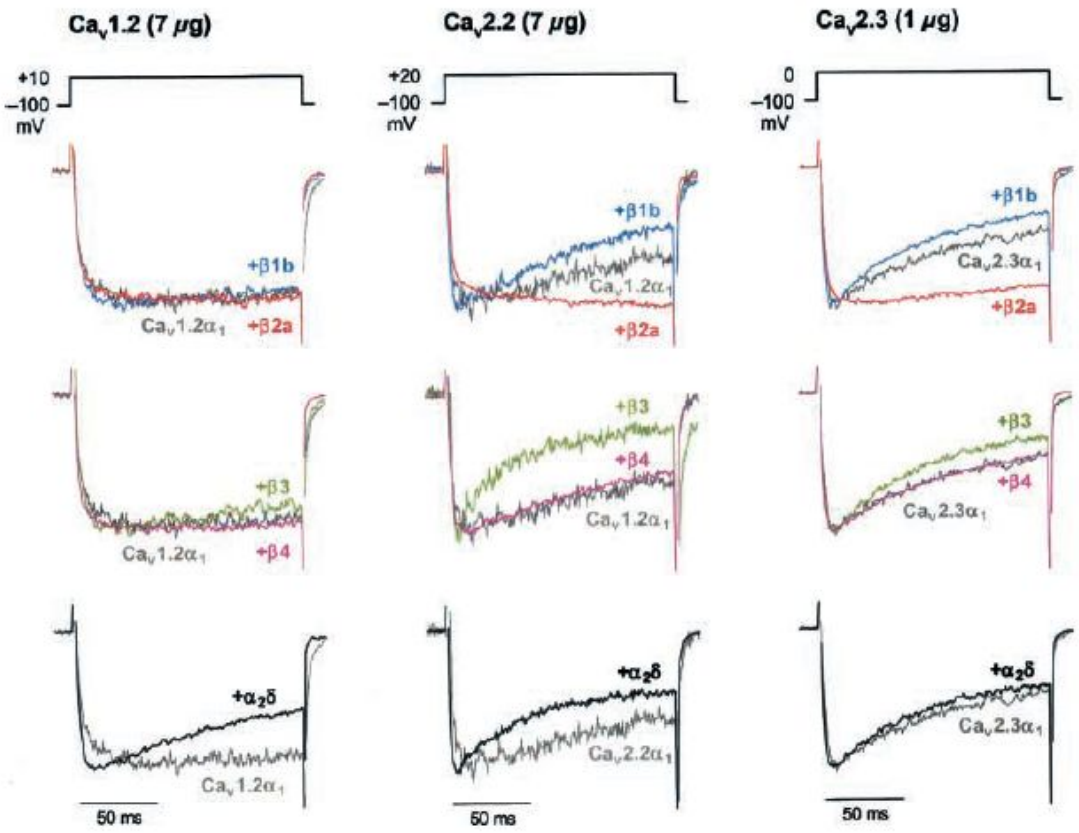

FIG. 3. Representative current traces obtained with $Ca_v1.2$, $Ca_v2.2$ and $Ca_v2.3$ calcium channels in the absence and the presence of various combinations of auxiliary α_2–δ_1 and β_{1b} subunits. The schematic at the top indicates the voltage step protocol. Current records are colour coded according to the subunit combination used in these experiments. Note that individual auxiliary subunits mediate qualitatively similar effects on the inactivation kinetics of the three types of calcium channel α_1 subunits.

The α_2–δ_1 subunit tended to accelerate inactivation kinetics of all calcium channels subtypes both in the absence and in the presence of β subunits. This is consistent with previous reports for $Ca_v1.2$ (Felix *et al*., 1997; Klugbauer *et al*., 1999), $Ca_v2.2$ (Gao *et al*., 2000) and $Ca_v2.3$ channels (Qin *et al*., 1998a) but not $Ca_v2.2$ channels in HEK-293 cells (Williams *et al*., 1992; Brust *et al*., 1993). Interestingly, α_2–δ subunit regulation of $Ca_v2.3$ channel inactivation was lost when the amount of $Ca_v2.3$ expression was reduced (compare Fig. 4C and D), and at this point, we do not have a convincing mechanistic explanation for this result. Interestingly, α_2–δ_1 could not accelerate inactivation of the three calcium channels when coexpressed with β_{2a}, suggesting that the β_{2a}-subunit-mediated slowing of inactivation dominates over α_2–δ_1-induced acceleration. Our collective data are again consistent with the idea that β subunit regulation may be specifically tailored to subsets of calcium channel subtypes in cooperation with α_2–δ_1 subunits.

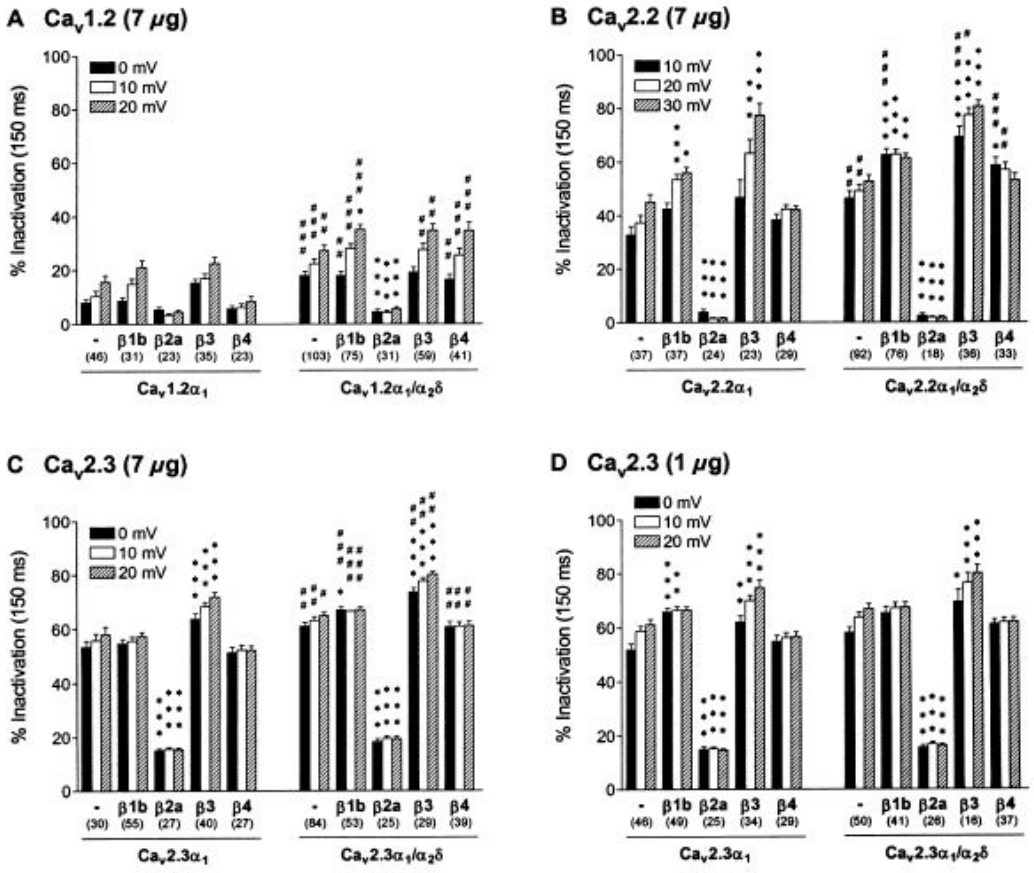

FIG. 4. Quantitative analysis of the effects of auxiliary subunits on the inactivation kinetics of $Ca_v1.2$ (A), $Ca_v2.2$ (B) and $Ca_v2.3$ (7 μg cDNA, C; 1 μg cDNA, D) calcium channels at three different test potentials. Error bars denote standard errors, numbers in parentheses reflect numbers of experiments. The asterisks and number symbols reflect statistical significance as described in detail in the Materials and methods section. The magnitude of the bars reflects the percentage of current that has inactivated over the course of a 150-ms test depolarization (see Spaetgens & Zamponi, 1999; Stotz & Zamponi, 2001).

Calcium channel β and α2–δ1 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. $Ca_v2.2$ (Fig. 5B) and $Ca_v2.3$ (Fig. 5C and D), but not $Ca_v1.2$ (Fig. 5A), exhibited distinguishable closed- and open-state inactivation in the absence of β subunits, as seen from the biphasic voltage-dependence of inactivation. The separation of closed- and open-state inactivation was augmented following coexpression of the β_{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 $Ca_v1.2 + \alpha_2$–δ_1 channels (see Table 1), coexpression with the β_{2a} subunit mediated a discernible 7 mV depolarizing shift in half-inactivation potential, consistent with previous findings (Olcese *et al*., 1994; Qin *et al*., 1998b; Gerster *et al*., 1999). By contrast, the remaining calcium channel β subunit isoforms tended to mediate hyperpolarizing shifts in half-inactivation potential, which in the case of $Ca_v2.3$ 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 *et al*., 1993; Wakamori *et al*., 1999; Canti *et al*., 2001). The α_2–δ_1 subunit mediated negative shifts in half-inactivation potential for $Ca_v1.2$, $Ca_v2.2$ and $Ca_v2.3$ (1 μg) channels, but the effects were typically not additive with those of the β subunits. This suggests that the α_2–δ subunit is capable of regulating the voltage-dependence of calcium channel inactivation by a mechanism that may perhaps converge with that of β subunit regulation. Altogether, however, with the exception of the β_{2a} subunit, auxiliary subunit regulation of half-inactivation potentials is relatively weak, with shifts in $V_{1/2,inact}$ of typically less than 10 mV.

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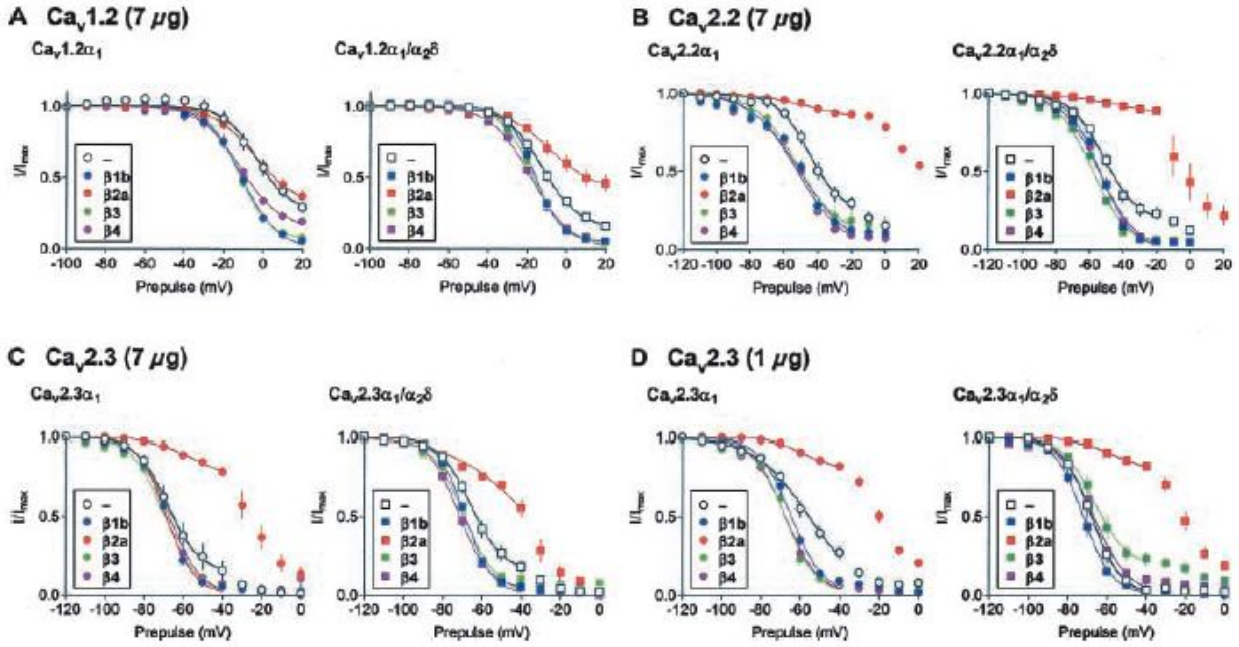

FIG. 5. Voltage dependence of inactivation in the form of steady-state inactivation curves obtained for $Ca_v1.2$ (A), $Ca_v2.2$ (B) and $Ca_v2.3$ (7 μg cDNA, C; 1 μg cDNA, D) in the absence and the presence of various auxiliary subunits. The individual data points are means of 5–37 experiments (see 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 $Ca_v1.2$ calcium channels

	Activation				Inactivation (steady-state)		
	$V_{1/2,act}$(mV)	E_{rev}(mV)	k_{act}(mV)	n	$V_{1/2,act}$(mV)	k_{act}(mV)	n
$Ca_v1.2\alpha_1$	5.9 ± 0.9	48.7 ± 1.0	7.7 ± 0.2	46	−3.6 ± 2.1	9.2 ± 1.3	19
$Ca_v1.2\alpha_1$/ β1b	6.4 ± 0.8	49.2 ± 0.9	7.2 ± 0.2	31	−12.9 ± 2.2***	7.3 ± 0.8	18
$Ca_v1.2\alpha_1$/ β2a	1.7 ± 0.7**	45.6 ± 1.4	6.8 ± 0.2*	23	−1.6 ± 2.1	12.1 ± 2.1	10
$Ca_v1.2\alpha_1$/ β3	6.7 ± 0.6	49.4 ± 0.6	7.3 ± 0.1	35	−12.6 ± 0.9***	8.2 ± 0.6	28
$Ca_v1.2\alpha_1$/ β4	0.6 ± 1.1***	48.4 ± 1.0	6.7 ± 0.2**	21	−11.3 ± 1.0*	10.3 ± 0.8	14
$Ca_v1.2\alpha_1$/ $\alpha_2\delta$	1.9 ± 0.4###	48.6 ± 0.4	6.8 ± 0.1###	103	−11.3 ± 1.3###	8.9 ± 0.4	37
$Ca_v1.2\alpha_1$/ $\alpha_2\delta$/ β1b	1.1 ± 0.5###	47.6 ± 0.3	6.1 ± 0.1***###	75	−16.7 ± 0.9*	6.8 ± 0.3	33
$Ca_v1.2\alpha_1$/ $\alpha_2\delta$/ β2a	−0.9 ± 1.0*	46.8 ± 0.8	6.8 ± 0.1	31	−4.4 ± 3.1*	12.8 ± 1.9*	12
$Ca_v1.2\alpha_1$/ $\alpha_2\delta$/ β3	2.5 ± 0.7###	48.3 ± 0.5	6.3 ± 0.2###	59	−17.5 ± 1.0**	8.2 ± 0.5	29
$Ca_v1.2\alpha_1$/ $\alpha_2\delta$/ β4	−0.9 ± 0.7*	46.0 ± 0.6*	6.3 ± 0.2	41	−19.2 ± 1.5**#	8.2 ± 0.2	20

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 $Ca_v2.2$ calcium channels

	Activation				Inactivation (steady-state)		
	$V_{1/2,act}$(mV)	E_{rev}(mV)	k_{act}(mV)	n	$V_{1/2,act}$(mV)	k_{act}(mV)	n
$Ca_v2.2\alpha_1$	133 ± 0.9	54.2 ± 1.1	5.9 ± 0.2	37	−42.9 ± 1.8	11.3 ± 11.7	14
$Ca_v2.2\alpha_1$/ β1b	14.0 ± 0.5	56.6 ± 0.8	5.7 ± 0.1	37	−50.9 ± 1.8**	14.4 ± 0.9	13
$Ca_v2.2\alpha_1$/ β2a	12.5 ± 1.1	58.4 ± 0.9	5.5 ± 0.1	24	ND	ND	16
$Ca_v2.2\alpha_1$/ β3	15.6 ± 1.0	51.8 ± 0.9	5.5 ± 0.2	23	−54.2 ± 1.2***	11.0 ± 1.4	7
$Ca_v2.2\alpha_1$/ β4	12.2 ± 0.6	58.1 ± 0.6	5.6 ± 0.1	29	−52.2 ± 1.2***	13.6 ± 0.4	12
$Ca_v2.2\alpha_1$/ $\alpha_2\delta$	13.1 ± 0.7	57.4 ± 0.7*	5.3 ± 0.2	92	−49.4 ± 1.0##	9.9 ± 0.6	31
$Ca_v2.2\alpha_1$/ $\alpha_2\delta$/ β1b	9.0 ± 0.6***###	57.7 ± 0.6	4.2 ± 0.2***###	76	−54.5 ± 1.0**	8.8 ± 0.0###	34
$Ca_v2.2\alpha_1$/ $\alpha_2\delta$/ β2a	9.0 ± 1.5	57.4 ± 1.6	4.5 ± 0.4	18	ND	ND	7
$Ca_v2.2\alpha_1$/ $\alpha_2\delta$/ β3	11.8 ± 0.9	58.9 ± 0.6###	4.7 ± 0.3	36	−58.5 ± 0.8***	10.2 ± 0.4	21
$Ca_v2.2\alpha_1$/ $\alpha_2\delta$/ β4	10.5 ± 1.0*	58.6 ± 1.0	4.4 ± 0.2*##	33	−54.6 ± 1.1 *	10.2 ± 0.3*	23

Asterisks and number symbols reflect statistical significance as described in the Materials and methods section.

TABLE 3. Effects of auxiliary subunits on activation and steady-state inactivation of $Ca_v2.3$ calcium channels

	Activation				Inactivation (steady-state)		
	$V_{1/2,act}$(mV)	E_{rev}(mV)	k_{act}(mV)	n	$V_{1/2,act}$(mV)	k_{act}(mV)	n
$Ca_v2.3\alpha_1$	−15.4 ± 1.6	49.0 ± 0.9	3.4 ± 0.3	30	−62.8 ± 3.6	7.4 ± 0.5	12
$Ca_v2.3\alpha_1$/ β1b	−20.8 ± 11.0*	47.8 ± 0.5	2.5 ± 0.2*	55	−68.9 ± 1.2	6.3 ± 0.1	14
$Ca_v2.3\alpha_1$/ β2a	−14.3 ± 1.3	50.2 ± 0.7	3.7 ± 0.1	27	ND	ND	12
$Ca_v2.3\alpha_1$/ β3	−11.3 ± 0.6	47.5 ± 0.7	4.6 ± 0.1**	40	−69.9 ± 0.7	7.7 ± 0.2	16
$Ca_v2.3\alpha_1$/ β4	−12.7 ± 0.9	50.1 ± 0.3	4.0 ± 0.1	27	−67.9 ± 0.9	7.0 ± 0.2	17
$Ca_v2.3$cti/ ct-.6	−16.1 ± 1.1	50.0 ± 0.5	2.8 ± 0.2	84	−62.5 ± 2.0	8.6 ± 0.6	27
$Ca_v2.3\alpha_1$/ $\alpha_2\delta$/ β1b	−20.6 ± 1.2**	48.8 ± 0.6	1.8 ± 0.2***	53	−70.5 ± 0.6*	7.4 ± 0.3	19
$Ca_v2.3\alpha_1$/ $\alpha_2\delta$/ β2a	−12.1 ± 1.0	50.0 ± 0.6	3.6 ± 0.1	25	ND	ND	10
$Ca_v2.3\alpha_1$/ $\alpha_2\delta$/ β3	−10.5 ± 0.7**	51.2 ± 0.0	4.1 ± 0.2***	29	−73.7 ± 0.6***	9.9 ± 0.2#	11
$Ca_v2.3\alpha_1$/ $\alpha_2\delta$/ β4	−14.2 ± 1.3	49.3 ± 0.6	3.3 ± 0.2	39	−72.8 ± 1.1***	7.5 ± 0.3	22
$Ca_v2.3\alpha_1$ (1 μg)	−7.2 ± 0.8	49.3 ± 0.7	4.8 ± 0.1	46	−59.0 ± 2.1	11.0 ± 1.0	10
$Ca_v2.3\alpha_1$ (1 μg)/ β1b	−11.1 ± 0.7*	47.7 ± 0.8	4.2 ± 0.1	49	−65.2 ± 1.5	7.2 ± 0.3***	11
$Ca_v2.3\alpha_1$ (1 μg)/ β2a	−8.4 ± 0.8	50.6 ± 11.1	4.4 ± 0.2	25	ND	ND	14
$Ca_v2.3\alpha_1$ (1 μg)/ β3	−8.0 ± 0.5	46.2 ± 0.9	4.6 ± 0.1	34	−68.5 ± 0.8***	7.1 ± 0.4***	12
$Ca_v2.3\alpha_1$ (1 μg)/ β4	−10.0 ± 0.6	49.9 ± 0.5	4.3 ± 0.1	29	−68.2 ± 1.0***	7.6 ± 0.3***	22
$Ca_v2.3\alpha_1$ (1 μg)/ $\alpha_2\delta$/ β1b	−7.7 ± 1.2	50.3 ± 10.7	4.4 ± 0.2	50	−68.6 ± 1.9##	6.3 ± 0.5###	5
$Ca_v2.3\alpha_1$ (1 μg)/ $\alpha_2\delta$/ β1b	−15.3 ± 1.5***#	49.4 ± 0.9	2.7 ± 0.3***###	41	−72.5 ± 1.9#	6.2 ± 0.3	8
$Ca_v2.3\alpha_1$ (1 μg)/ $\alpha_2\delta$/ β2a	−9.2 ± 0.8	51.8 ± 0.6	4.1 ± 0.1	26	ND	ND	14
$Ca_v2.3\alpha_1$ (1 μg)/ $\alpha_2\delta$/ β3	−6.7 ± 0.9	49.2 ± 1.1	4.7 ± 0.1	16	−66.0 ± 2.2	7.9 ± 0.7	4
$Ca_v2.3\alpha_1$ (1 μg)/ $\alpha_2\delta$/ β4	−10.8 ± 0.8	50.1 ± 0.7	3.8 ± 0.2	37	−68.4 ± 1.8	7.1 ± 0.4	7

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_{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_{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 α_2–δ_1, calcium channel β subunits did not affect the whole cell conductance of $Ca_v1.2$ channels. For $Ca_v2.2$ and $Ca_v2.3$ (1 μg) channels lacking α_2–δ_1, coexpression of β subunits significantly affected median values, but not the overall means and, strikingly, coexpression with β_3 never resulted in current enhancement. Finally, for $Ca_v1.2$ channels, the coexpression of α_2–δ_1 significantly increased whole cell conductance without further enhancement of current activity by β subunit coexpression. By contrast, whereas the presence of α_2–δ_1 also enhanced $Ca_v2.2$ 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 α_2–δ_1 and β subunits act synergistically to promote $Ca_v2.2$ activity. A similar trend was also observed with $Ca_v2.3$, here mainly β_{1b} and β_4 appeared to act in concert with α_2–δ_1. 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 α_2–δ_1 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 α_2–δ_1 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 β_3 subunits did not affect current levels, but mediated a pronounced effect on inactivation kinetics).

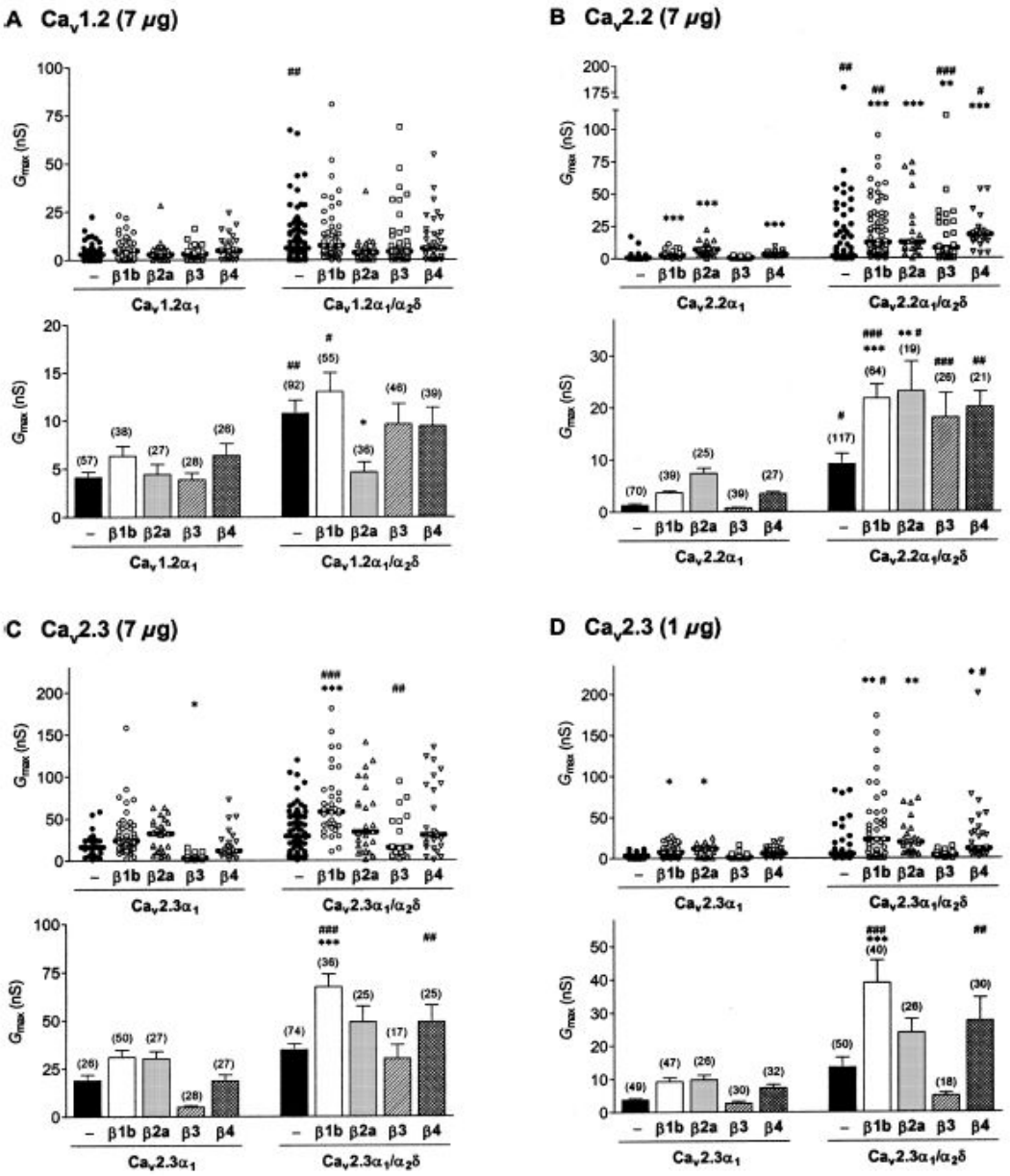

FIG. 6. Effects of subunit coexpression on whole cell conductance (G_{max}) of tsA-201 cell expression $Ca_v1.2$ (A), $Ca_v2.2$ (B) and $Ca_v2.3$ (7 μg cDNA, C; 1 μg cDNA, D) in the absence and the presence of various auxiliary subunits. G_{max} values were obtained from Boltzmann fits to individual current voltage relations. In each case, data from all GFP-positive cells were included, even if no detectable current was present, and for cells with peak currents smaller than 10 pA, G_{max} values were set arbitrarily to zero as we were unable to fit current voltage relations properly for such small currents. The upper panels show all of the individual data points, and the median is shown as a solid line. The bar graphs in the bottom panel reflect means plus standard errors. Numbers in parentheses indicate the numbers of experiments. The asterisks and number symbols indicate statistical significance as outlined in the Materials and mMethods section. In the upper panels, statistical significance refers to the median values (Kruskal–Wallis test with Dunn's post test). In the lower panels, mean G_{max} values were assessed via one-way ANOVAs with Dunnett's post test.

Figure 7 examines the fraction of GFP-positive cells that did not express detectable membrane currents. For $Ca_v1.2$ calcium channels (Fig. 7A), we typically observed an ~80% success rate, irrespective of the subunit that was coexpressed (with exception of β_{1b}, which appeared to increase the fraction of expressing cells further). Hence, the observation that β subunits did not regulate current densities (Fig. 6A) is not secondarily due to an increase in cells with no detectable current. With $Ca_v2.3$ 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_{max} values observed with this $Ca_v2.3$. The data obtained with $Ca_v2.2$ are perhaps the most striking. In the absence of β subunits, the percentage of cells with no detectable current was ~45% and ~35% in the absence and the presence of α_2–δ_1,

respectively (Fig. 7B). Coexpression with β_{1b}, β_{2a} or β_4 subunits dramatically enhanced the fraction of expressing cells, whereas β_3 subunits did not. These data indicate that the small enhancements of median G_{max} values in the absence of α_2–δ_1 in Fig. 6B were, at least in a part, due to increased success rate. The finding that > 95% success rate was seen with $Ca_v2.2$ channels in the presence of β_{1b}, β_{2a} and β_4 irrespective of the presence of α_2–δ_1 indicates that the increase in G_{max} in the concomitant presence of α_2–δ_1 and either one of these β 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 $Ca_v2.2\alpha_1$ alone or with α_2–δ_1 were negligible and the percentages of detectable current-expressing cells were less than 10% in both cases. Cells cotransfected with $Ca_v2.2\alpha_1$ and β_{1c} showed robust currents and the current-expressing cells were increased up to ~35%. This percentage was not changed by further cotransfection of α_2–δ_1 with $Ca_v2.2\alpha_1$ and β_{1c}, although current amplitude was enormously enhanced due to increase in the number of large current cells.

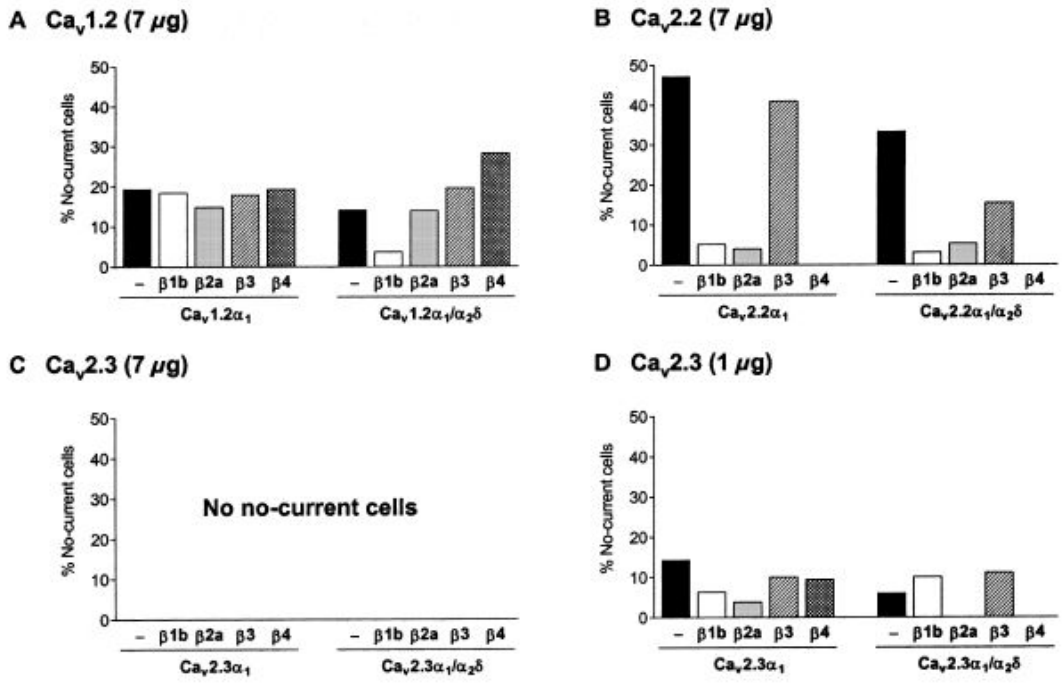

FIG. 7. Percentage of GFP-positive cells that did not express detectable currents following expression of $Ca_v1.2$ (A), $Ca_v2.2$ (B) and $Ca_v2.3$ (7 μg, C; 1 μg, D) calcium channels alone or in the presence of various auxiliary subunits.

Collectively, β_{1b}, β_{2a} and β_4 subunits are capable of increasing the fraction of cells that express functional currents as well as current amplitude of Ca_v2 channels, especially $Ca_v2.2$. By contrast, α_2–δ_1 subunits solely enhance current amplitude without affecting the fraction of cells expressing detectable barium currents. Pronounced β subunit effects on the fraction of expressing cells may contribute to synergistic effects between β and α_2–δ_1 subunits on $Ca_v2.2$ 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 α_1), plus several lower molecular weight auxiliary subunits (for a review see Catterall, 2000). Early studies in *Xenopus* oocytes indicated that expression of the α_1 alone subunit can result in functional channels, but that coexpression with β 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 β subunits, four different types of α_2–δ and eight different isoforms of γ subunits. The mRNA or protein expression of α_1, β and α_2–δ 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 α_2–δ and β are *bona fide* subunits of neuronal HVA calcium channels. In particular, the mutual interaction sites for binding between α_1 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 Ca_v3 family and $Ca_v1.4$ channels (McRory *et al*., 2004), all known α_1 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 α_2–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 α_1 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, β_1 and β_3 subunits have been reported to accelerate inactivation, and to mediate substantial hyperpolarizing shifts in the voltage-dependences of activation and inactivation, whereas β_{2a} 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, α_2–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 α_2–δ subunits mediated little, if any, effect on the voltage dependence of activation for the three channel types examined. For $Ca_v1.2$, $Ca_v2.2$ and $Ca_v2.3$ 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 β_{1b}, β_{2a} and β_3 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 β_{2a} subunits appeared to mediate dramatic effects on steady-state inactivation, notably by increasing the fraction of open state inactivation of Ca_v2 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 α_2–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 β_1, β_3 and β_4 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 α_1/β/α_2–δ combinations on slow inactivation in our system. Finally, for $Ca_v1.2$ 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 $Ca_v1.2$ channel currents by α_2–δ_1 in HEK-298 cells (Bangalore *et al*., 1996), the α_2–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 α_2–δ, β_3 or β_{2a} subunits resulted in robust and highly reproducible effects on inactivation rates, indicating that these subunits were

functionally expressed in our experiments. The expression of β_{1b} subunits resulted in a significant increase in G_{max} for $Ca_v2.2 + \alpha_2–\delta_1$ and $Ca_v2.3 + \alpha_2–\delta_1$ calcium channels and substantially increased the fraction of $Ca_v2.2$-expressing cells. In addition, β_{1b} protein expression is readily confirmed via Western blots (S. E. Jarvis *et al.*, unpublished observations). Finally, although coexpression with β_4 subunits mediated little effect on calcium channel function, this subunit also potently increased the fraction of $Ca_v2.2$-expressing cells, and we have demonstrated recently that $Ca_v2.2$ effectively coimmunoprecipitate with β_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 α_1 subunit to allow its efficient translocation to the plasma membrane (Bichet *et al*., 2000). However, trafficking of the α_1 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 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 $Ca_v1.2$ channel activity, and that they mediate a less than two-fold increase in G_{max} for $Ca_v2.3$ 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 $Ca_v1.2$ current density is due to lack of β subunit expression, because all four β subunits were able to regulate inactivation kinetics of $Ca_v2.2$ and $Ca_v2.3$ channels, in addition to affecting the position of the steady-state inactivation curve of $Ca_v1.2$ 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 $Ca_v2.2$ calcium channels (Feng *et al*., 2001). Moreover, there is compelling evidence that β and $\alpha_2–\delta$ subunits are critical for neuronal and/or muscle physiology. A point mutation leading to a premature stop codon in the mouse β_4 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 β_3 subunit reduces L-type and N-type channel activity in neurons (Namkung *et al*., 1998), and knockdown of the calcium channel β_{1a} subunit prevents excitation contraction coupling and alters L-type calcium channel kinetics (Gregg *et al*., 1996). Mice lacking β_{2a} die during embryogenesis due to absence of heartbeat; however, restoration of β_{2a} expression in the heart rescues the lethal phenotype such that homozygous mice lacking β_{2a} in other organs (including the brain) are behaviorally normal (Ball *et al*., 2002). A premature stop codon in the $\alpha_2–\delta$ 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 $Ca_v1.2$ and $Ca_v2.3$ 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 (Wittemann *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 $\alpha_2–\delta_1$ 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.

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