The $\alpha_2\delta$ subunit augments functional expression and modifies the pharmacology of CaV1.3 L-type channels

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**Abstract**

The auxiliary CaV$\alpha_2\delta$-1 subunit is an important component of voltage-gated Ca$^{2+}$ (CaV) channel complexes in many tissues and of great interest as a drug target. Nevertheless, its exact role in specific cell functions is still unknown. This is particularly important in the case of the neuronal L-type CaV channels where these proteins play a key role in the secretion of neurotransmitters and hormones, gene expression, and the activation of other ion channels. Therefore, using a combined approach of patch-clamp recordings and molecular biology, we studied the role of the CaV$\alpha_2\delta$-1 subunit in the functional expression and the pharmacology of recombinant L-type CaV1.3 channels in HEK-293 cells. Co-expression of CaV$\alpha_2\delta$-1 significantly increased macroscopic currents and conferred the CaV1.3(3α1/CaV1.3δ) channels sensitivity to the antiepileptic/analgesic drugs gabapentin and AdGABA. In contrast, CaV$\alpha_2\delta$-1 subunits harboring point mutations in N-glycosylation consensus sequences or the proteolytic site as well as in conserved cysteines in the transmembrane δ domain of the protein, reduced functionality in terms of enhancement of CaV 1.3(3α1/CaV1.3β) currents. In addition, co-expression of the δ domain drastically inhibited macroscopic currents through recombinant CaV1.3 channels possibly by affecting channel synthesis. Together these results provide several lines of evidence that the CaV$\alpha_2\delta$-1 auxiliary subunit may interact with CaV1.3 channels and regulate their functional expression.

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1. Introduction

L-type voltage-gated Ca$^{2+}$ (CaV) channels are expressed in many different cell types and tissues. In myocytes they are essential for excitation–contraction coupling, whereas in neurons and endocrine cells they regulate neurotransmitter and hormone release, gene expression, and the activity of other ion channels [1,2]. Biochemical evidence suggests that L-type CaV channels are comprised of five subunits. A principal transmembrane $\alpha_1$ subunit is predicted to associate with a disulfide-linked $\alpha_2\delta$ dimer, an intracellular $\beta$ subunit, and a transmembrane $\gamma$ subunit [2,3]. The $\alpha_1$ subunit is the ion-conducting element in the channel protein complex [2,3].

Four mammalian genes encode L-type CaV1 channel $\alpha_1$ subunits: CaV1.1 (also known as $\alpha_1G$), CaV1.2 ($\alpha_1C$), CaV1.3 ($\alpha_1D$), and CaV1.4 ($\alpha_1F$). CaV1.1(1α1C), CaV1.1(1α1G), and CaV1.4(1α1F) subunits are enriched in skeletal muscle and retina, respectively, whereas CaV1.2(1α1C) and CaV1.3(3α1) subunits are expressed in many cells including neurons and endocrine cells [1,2]. CaV1.2 and CaV1.3 channels underlie the majority of L-type currents in neuronal, endocrine, and cardiovascular systems. CaV1.3 channels have relatively low activation thresholds, are less sensitive to dihydropyridine antagonists, and activate with fast kinetics when compared to CaV1.2 L-type currents [1]. There is growing interest in CaV1.2 and CaV1.3 channels because of their link to neurodegenerative disorders including autism, bipolar disorder, and Parkinson’s disease [4–8]. Understanding the mechanisms that regulate L-type calcium channel activity and surface expression is of major importance.

In the central nervous system, L-type Ca$^{2+}$ channels (CaV1.2 and CaV1.3) apparently do not support synaptic transmission, but seem to play an important role in the excitation–transcription coupling. It has been reported that Ca$^{2+}$ entry via postsynaptic L-type channels activates transcription factors pCREB [9,10] and NFATc4.
Phosphorylation of CREB, acting in conjunction with nuclear translocation and co-activator proteins promotes transcription of multiple genes [12,13]. Likewise, CaV 1.3 channels play a unique role in hearing. Inner hair cells (IHCs), the primary sensory receptors of the mature mammalian cochlea, are responsible for relaying acoustic information transduced by mechano-sensitive channels to the central nervous system via afferent auditory nerve fibres. This is driven by Ca2+ entering IHCs through L-type channels of the CaV1.3 class [14] activated in response to depolarizing receptor potentials initiated by hair bundle deflection. In addition, it has been reported that CaV1.3 L-type channels are important for the sinoatrial node function. Using gene-targeted deletion of the CaV1.3 subunit, Zhang et al. [15] found a decrease in the rate of firing associated with a diminished rate of diastolic depolarization. Last, CaV1.3 L-type channels are expressed at high density and play a role in the control of hormone secretion in a variety of endocrine cells including pancreatic β- and adrenal chromaffin cells where they control insulin and catecholamine release [2,16].

In contrast to the functional studies, the molecular architecture of the L-type CaV1.3 channels is largely unknown. Though a role for the CaVβ subunits in determining a functional interaction with protein kinase A [17] and arachidonic acid [18] has been reported, little is known regarding the role of the Ca2+-channel auxiliary subunits in the regulation of the CaV1.3 channel activity. On the other hand, it has been reported that CaVαδ subunits promote surface expression of different CaVα1 subunits and it speeds channel activation and inactivation kinetics [19–30]. However, investigations of the CaVαδ subunit effects on CaV1.3 channels are lacking. Here, we show that CaVαδ augments surface expression of recombinant CaV1.3 channels in HEK-293 cells. We also show that co-expression of the CaVαδ subunit renders the CaV1.3 channels sensitive to antiepileptic/analgésic gabapentinoid drugs (GBP and AdGABA) and that co-expression of the transmembrane δ domain alone, together with the CaV1.3/CaVβ3 Ca2+ channel combination in absence or presence of CaVαδ, results in an important inhibition of the whole-cell current.

2. Materials and methods

2.1. Materials

Chloroquine (C-6628) and Fillipin III (F-4767) were obtained from Sigma–Aldrich (St. Louis, MO) and prepared as stock according to the manufacturer’s instructions. Gabapentin (1-aminomethyl)cyclohexane acetic acid; Neurontin®; Pfizer, New York, NY) and AdGABA (a generous gift of Drs. G. Zoidis and N. Kolocouris, University of Athens) were prepared as stock in distilled water and aliquots were stored at −20 °C. All other chemicals were of reagent grade and obtained from different commercial sources.

2.2. cDNA clones

Cell expression constructs were made by standard techniques and their fidelity was verified by DNA sequencing. The rat neuronal CaV 1.3α1 (GenBank accession number AF370009 [31]) was cloned into the pcDNA6/His vector (Invitrogen; Carlsbad, CA). The rabbit CaVβ1a subunit (M25817) was cloned in the pKCRH2 vector [32] while the cDNAs coding the rat brain CaVβ2a (M80545), CaVβ3 (M88751) and CaVβ4 (L02315) subunits were cloned into the pcDNA3 vector (Invitrogen). We also used the recombinant bistricomponent expression plasmid PIERESαδ/β2 [33], which carried the entire protein-coding region for the rat brain CaVαδ-1b Ca2+ channel auxiliary subunit (M86621), or its mutant constructs [27,33], and for the green fluorescent protein (GFP) coupled by an internal ribosomal entry site (IRES) sequence. The cDNA coding the CaVδ subunit, which was made by assembling a PCR fragment after the CaVαδ-1b signal sequence [22], and the CD8 surface marker were cloned in the pcDNA3 expression plasmid (Invitrogen). The PERK mutant constructs [34] were cloned into the pcDNAI/Amp vector (Invitrogen).

2.3. Site-directed mutagenesis

The Pfu DNA polymerase was used in all PCRs to generate the CaVαδ-1 mutations and all constructs were verified by sequencing. The glycosylation and proteolysis mutant constructs were generated following standard procedures in use in the laboratory [27,33]. The CaVαδ-1 subunit in which all cysteines in the extracellular region of δ were mutated to serines, was made using the pIREShrGFP-1a-based construct encoding the rat CaVαδ-1 [27] as a template and standard PCR techniques. In all cases, the mutations were introduced with 40-mer synthetic oligonucleotides using the Quick-Change XL II mutagenesis kit (Stratagene).

2.4. Cell culture and transfection

Human embryonic kidney (HEK)-293 cells (American Type Culture Collection, ATCC; Manassas, VA) were grown in Dulbecco’s modified Eagle’s medium (DMEM)-high glucose supplemented with 10% horse serum, 2 mM L-glutamine, 110 mg/l sodium pyruvate, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% CO2/95% air humidified atmosphere. After splitting on the previous day and seeding at ~60% confluence, cells were transfected with the cDNA clones mentioned earlier using the Lipofectamine Plus reagent (Invitrogen) according to the manufacturer’s instructions. After DNA–lipid complexes were allowed to form, cells were transfected with either cDNAs encoding CaV1.3α1 alone (1 μg DNA/35-mm culture dish) or co-transfected with cDNAs for CaVβ, CaVαδ-1 and CaVδ subunits in a 1:1 molar ratio (except where indicated).

The HEK-293 cell line stably expressing the CaV3.2 channel (GenBank accession number AF051946 [35]) was grown as previously described [36]. Likewise, the RIN-m5F insulinoma β-cells (ATCC) were cultured in RPMI-1620 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Both cell lines were plated on poly-L-lysine (0.05%)-precoated glass coverslips placed into 35-mm culture plates, and 24 h later were transfected with the CaVαδ-1 or δ subunit cDNA constructs using Lipofectamine Plus reagent (Invitrogen). 48 h after transfection, cells were subjected to electrophysiological recording.

2.5. Electrophysiology

Ionic currents were recorded using the whole-cell configuration of the patch-clamp technique [37], using an Axopatch 200B patch-clamp amplifier (Molecular Devices, Foster City, CA) and acquired on-line using a Digidata 1320A interface with pClamp8 software (Molecular Devices) as described elsewhere [27,33]. The offset potential between the pipette and bath solutions was zeroed prior to seal formation. After establishing the whole-cell mode, capacitive transients were cancelled with the amplifier. Series resistance values were typically 2–10 MΩ, and no records were used in which the voltage error (as defined by ΔV = IMAX × R0) was greater than 5 mV. Leak and residual capacitance currents were subtracted on-line by a P/4 protocol. Current signals were filtered at 2 kHz (internal 4 pole Bessel filter) and digitized at 5.71 kHz. Membrane capacitance (Cm) was determined as described previously [38] and used to normalize currents. The recording solutions are given in Table 1. Experiments were performed at room temperature (∼22 °C).
Table 1

Recording solutions. Units are in mM. The pH was adjusted to 7.3 with KOH (A–C) and CsOH (D and E).

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<th>TEA-Cl</th>
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<th>CsCl</th>
<th>MgCl₂</th>
<th>KCl</th>
<th>K-Asp</th>
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2.6. Semi-quantitative Western blot

Cells were mechanically detached from culture dishes, washed twice with PBS pH 7.4 (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄) and lysed in triple-detergent buffer containing proteases inhibitors (50 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1% SDS, 1.0% NP-40, 0.5% sodium deoxycholate, 1 mM PMSF, complete 1X; Roche Diagnostics). Lysis was performed in ice for 20 min vortexing each 5 min. The extracts were centrifuged to remove insoluble debris (10 min: 7500 \( \times \) g) and protein concentration in the supernatants was determined using Bradford assays. Samples with 50 \( \mu \)g of protein were boiled for 5 min in protein loading buffer (50 mM Tris–HCl [pH 6.8], 2% SDS, 10% glycerol, 5% \( \beta \)-mercaptoethanol, 0.01% bromophenol blue). Proteins were resolved in 10% SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Amersham Biosciences; Piscataway, NJ). After blocking with 6% non-fat dry milk in TBS-T (10 mM Tris–HCl, 0.15 M NaCl, 0.05% Tween-20), membranes were incubated overnight with the primary anti-CaV 1.3 antibody (Alomone Labs; Jerusalem, Israel) (1:600 in blocking solution). Membranes were then washed and incubated with horseradish peroxidase goat anti-rabbit secondary antibody (Invitrogen) diluted in TBS-T with 6% non-fat dry milk and developed with the ECL reagent (Amersham Biosciences). For \( \beta_3 \), proteins were blotted onto nitrocellulose membranes and developed with enhanced chemiluminescence as previously described [22,39]. As a protein loading control, membranes were stripped and incubated with a mouse monoclonal anti-\( \beta \)-actin antibody [40]. Semi-quantitative analysis was carried out by densitometry using the Kodak digital Science ID v.2.0 system program.

2.7. Data analysis

Curve fitting and statistical analyses were carried out using the SigmaPlot 10 software package (SPSS Inc.; Chicago, IL). The significance of observed differences was evaluated by Student’s unpaired t test. A probability less that 5% was considered to be significant. All experimental values are given as means ± S.E.M. The peak current values were converted to peak conductance values using the expression:

\[
G = \frac{I}{(V_m - V_{rev})}.
\]

where \( I \) is current, \( G \) is conductance, \( V_m \) is the test potential and \( V_{rev} \) is the extrapolated reverse potential. Conductance–voltage (\( G-V \)) curves for activation were fit with a Boltzmann equation of the form:

\[
G = \frac{G_{max}}{1 + \exp \left( \frac{V_m - V_{1/2}}{k} \right)}
\]

where \( G_{max} \) is maximum conductance, \( V_m \) is the test potential, \( V_{1/2} \) is the potential for half-maximal activation of \( G_{max} \) and \( k \) is a slope factor.

Fig. 1. CaV 1.3 channel regulation by CaV \( \beta \) and CaV \( \alpha_2\beta \) auxiliary subunits. The upper panels show representative traces of macroscopic \( \text{Ba}^{2+} \) current (\( I_{Ba} \)) recorded from HEK-293 cells that expressed CaV 1.3 channels in association with the CaV \( \beta \) subunit (\( \beta_1, \beta_2, \beta_3 \) or \( \beta_4 \)) in absence and presence of the CaV \( \alpha_2\beta \) subunit, using solutions A (external) and D (internal) [Table 1]. Currents were elicited by a depolarizing pulse to \( -30 \) mV from a \( V_h \) of \( -80 \) mV. The lower panels show average \( I-V \) relationships for \( I_{Ba} \) recorded from cells expressing CaV 1.3/\( \beta \) channels in absence and presence of the CaV \( \alpha_2\beta \) subunit (\( n = 9-50 \) cells). Currents were evoked by 10-mV depolarizing steps from a \( V_h \) of \( -80 \) mV to potentials between \(-70\) and \(+60\) mV.
HEK-293 cells expressing CaV 1.3 (panel B), respectively (see Table 1), and were elicited by 10-mV depolarizing steps from a construct lacking the conserved cysteines of the extracellular region of glycosylation of the V2 domain and disulfide bond formation between α, y and proteolytical cleavage to acquire its mature form. Three different CaVαβδ-1 mutant subunits were used in this work: αβδ (DM) has two point mutations in N-glycosylation sites; αβδ (P6) has a mutation in the putative site of proteolytical processing and a construct lacking the conserved cysteines of the extracellular region of δ (C6). (C) Steady-state inactivation curves were fitted with a Boltzmann function:

\[ I = \frac{I_{\text{max}}}{1 + \exp\left(\frac{V_m - V_{1/2}}{k}\right)} \]  

where the current amplitude I has decreased to a half-amplitude at \( V_{1/2} \) with an e-fold change over k mV.

Current activation and decay were fitted with a second order exponential equation of the form:

\[ I = A_{\text{fast}} \exp\left(-\frac{t}{\tau_{\text{fast}}}\right) + A_{\text{slow}} \exp\left(-\frac{t}{\tau_{\text{slow}}}\right) \]  

where \( t \) represents the time after the onset of the test pulse, \( A_{\text{fast}} \) and \( A_{\text{slow}} \) are the contribution of a fast and a slow component to the total current amplitude, and \( \tau_{\text{fast}} \) and \( \tau_{\text{slow}} \) are the time constants associated with each component.

### 3. Results

#### 3.1. The CaVα2δ-1 subunit increases macroscopic Ca\(^{2+}\) currents in cells expressing recombinant CaV 1.3 channels

We co-expressed CaVα2δ-1 and CaV 1.3 subunits together with different CaVβ subunits in HEK-293 cells to establish if CaVα2δ-1 plays a role in regulating expression levels of CaV 1.3 L-type channels. Ca\(^{2+}\) channel currents in cells expressing CaVα2δ-1 were substantially greater (3–5-fold) than those in cells lacking exogenous CaVα2δ-1. This effect of CaVα2δ-1 occurred regardless of CaVβ subunit type and was independent of test voltage (Fig. 1). Given that Western blot experiments have shown that untransfected HEK-293 cells do not express endogenous CaVα2δ-1 [33], CaV 1.3/CaVβ current enhancement detected after transfection may be therefore considered to be mediated by the heterologously expressed CaVα2δ-1 subunit. Likewise, our subsequent investigations of CaVα2δ-1 focused on the CaV 1.3/CaVβ combination because these two subunits tend to co-exist in neuronal and neuroendocrine tissue [41,42].
We next used more physiological recording conditions to test if CaVα2δ-1 augmented CaV1.3 currents when calcium is the charge carrier (Supplemental Fig. 1). L-type currents recorded with Ca2+ inactivate during the test pulse because they undergo pronounced Ca2+-dependent inactivation [43,44]. In cells expressing the CaVα2δ-1 subunit, L-type currents exhibited more prominent Ca2+-dependent inactivation at voltages > −40 mV reflecting greater levels of Ca2+ entry [45,46]. Under these recording conditions, CaVα2δ-1 still increased L-type current density over a range of voltages and without affecting the voltage dependence of channel activation and steady-state inactivation.

3.2. Effect of over-expression of CaVα2δ-1 mutant constructs on recombinant CaV1.3α1/β3 channels

We were interested in knowing if the stimulatory effects of CaVα2δ-1 on CaV1.3 channels are similar to its reported effects on CaV2.2. As mentioned earlier, CaVα2δ-1 is a glycosylated polypeptide that possesses a single transmembrane domain (δ) with a short intracellular C terminus and a long extracellular portion (Fig. 2A) which serves as an anchor in the cell membrane [2,3,47]. We know that N-glycosylation at N136 and N184 and proteolytic processing at amino acid residues 941–946 of CaVα2δ-1 are important for its stimulatory effects on neuronal CaV2.2 channels [27,33]. We therefore asked if the same sites play a role in CaVα2δ-1 mediated stimulation of CaV1.3 channels using different mutant constructs (Fig. 2B). As can be seen in Fig. 2C, the stimulatory effects of CaVα2δ-1 were partially lost in the double N-glycosylation CaVα2δ-1 mutant, N136Q and N184Q [33], and completely abolished in the proteolytic-site truncated CaVα2δ-1 mutant, P6 [27]. It is worth noting that previous results in our laboratory indicate that both mutant constructs are expressed in the HEK-293 cells at similar levels than the wild-type CaVα2δ-1 protein [27,33].

We also tested a CaVα2δ-1 construct in which we mutated all six conserved cysteine residues in the δ domain (C6), to prevent association with α2. The C6 CaVα2δ-1 mutant was also unable to augment CaV1.3 channel currents (Fig. 2D) implying that association between α2 and δ subunits is needed for its effects on current density. As with the double N-glycosylation and proteolytic-site mutated versions of CaVα2δ-1, the C6 mutant is not express at a significantly different level than wild-type in the HEK-293 cells (data not shown).

3.3. The δ domain attenuates CaV1.3α1/β3 channel expression

Although the actions of the CaVα2δ-1 subunit have been probed using various glycosylation and deletion mutants, the effect of the δ domain in isolation from α2 is still virtually unexplored [22,48]. We therefore assessed the effect of the δ domain on CaV1.3 channel currents. L-type currents in cells expressing the δ subunit were significantly smaller compared to currents in control cells over a range of test voltages (Fig. 3A and B). We also compared currents in...
cells co-expressing δ with those lacking δ and those expressing an unrelated control protein CD-8 (Fig. 3C). Likewise, we found that the inhibitory effects of δ were independent of recording conditions and observed when Ca$^{2+}$ or Ba$^{2+}$ were used as charge carriers (Supplemental Fig. 2).

The effects of δ reported here may be the result of a long-term regulation. It is possible that the Ca$_{\delta}$δ subunit affects processes that control surface targeting and/or overall levels of CaV1.3 protein. To test if the inhibitory effects of δ involved internalization, we used chloroquine, a lysosomal inhibitor, filipin III, a raft/caveolae-dependent endocytosis inhibitor and MG-132, a selective inhibitor of the 26S proteasome. Neither internalization inhibitor interfered with the actions of δ on L-type current density (Fig. 3D).

The inhibitory effects of the δ domain on current density depended on cDNA concentrations. Using fixed levels of CaV1.3α and CaVβ3 cDNAs (1:1 molar ratio) we varied δ cDNA levels and show a dose dependent decrease in L-type current densities with an increase in the relative molar ratio of δ (Fig. 4A and B). Interestingly, wild-type CaVα2δ-1 could not compete away the inhibitory effects of δ (Fig. 4C and D). L-type currents measured in cells co-expressing (CaV1.3α/CaVβ3/CaVδ) were attenuated greatly in the presence of δ, suggesting a possible interaction of δ with the channel complex. Likewise, the inhibitory action of δ was channel specific inhibiting CaV2.2 (Supplemental Fig. 3) and CaV1.3 channels (Fig. 4A–C) but not affecting low voltage-activated CaV3.2 (T-type) currents or endogenous K$^+$ currents recorded in untransfected HEK-293 cells (Fig. 4E and F).

3.4. δ Decreases CaV1.3 channel expression

We next quantified the levels of CaV1.3α and CaVβ3 subunits in cells expressing and lacking δ by Western blotting using CaV1.3 and CaVβ3 specific antibodies. Levels of both CaV1.3α and CaVβ3 subunits, but not control CD8 protein were significantly lower in cells expressing δ compared to control cells (Fig. 5). The δ-dependent decrease in CaV1.3 and CaVβ3 protein levels might not involve the unfolded protein response (UPR) pathway which has been implicated in the mechanism of action of hemi-Ca$^{2+}$ channels and Ca$^{2+}$ channel-related subunits [49–51], given that two mutant kinase-lacking PERK constructs, PERK ΔC and PERK K618A [34], that interfere with this pathway apparently did not prevent the inhibitory effects of δ on CaV1.3 L-type currents (Fig. 3D).

3.5. Native L-type current are regulated by wild-type α2δ-1 and isolated δ subunits

We next asked if CaVα2δ-1 could also influence native CaV1.3 L-type channels and used RIN-m5F rat insulinoma β-cells. Transient
expression of exogenous wild-type CaVα2δ-1 and δ in RIN-m5F cells resulted in significantly larger and smaller currents densities, respectively, when compared to control L-type currents supporting the use of cellular systems over-expressing the CaV1.3 channel types to the drug.

On the other hand, GBP did influence the voltage dependence of CaV1.3α1(CaVβ3/CaVα2δ-1) channel activation as well as the kinetics of activation and inactivation. GBP induced ~10 mV right shift in the voltage dependence of L-channel activation, increased the rate of channel activation, and decreased the rate of the slow component of channel inactivation (Fig. 7B). The effect of GBP on the slow component of L-channel inactivation is consistent with its effects on other (N- and P/Q-type) Ca2+ channels [30,57].

Finally, we examined the effects of 2-aminomethyl-2-tricyclo[3.3.1.11,7]decaneacetic acid hydrochloride 5 (AdGABA), a novel adamantine derivative of GABA that also has strong inhibitory effects on recombinant N-type channels [54]. It is worth noting that although the pharmacological evaluation of AdGABA has demonstrated anticonvulsive and antinociceptive properties, these properties were detectable only at high (sedative) doses, which may limit its potential clinical use. However, both gabapentin (GBP) and AdGABA seem to be acting via the same mechanism [54].

Chronic exposure (48 h) to 1 mM AdGABA strongly inhibited peak L-type current amplitudes in cells expressing CaV1.3α1/CaVβ3 with CaVα2δ-1 (~2.2-fold inhibition) but not in cells without CaVα2δ-1 (Fig. 7C). AdGABA did not affect the voltage dependence or rate of channel activation but like GBP, it lengthened the slow component of channel inactivation in cells expressing CaVα2δ-1 (Fig. 7D).

The pharmacological changes in CaV1.3 L-channels mediated by CaVα2δ-1 are mechanistically similar to those on CaV2.2 N-type channels, but the channels differ in their pharmacological specificity for GBP and AdGABA. Preferential action on CaV2.2 channels by GBP (Fig. 8) might explain why this drug is an effective analgesic.

4. Discussion

Although neuronal L-type Ca2+ channels are thought to open too slowly to contribute to action potential-dependent Ca2+ entry, they seem to play an essential role in regulating activity-dependent gene expression. A complication of studying native L-type channels is that they represent a minor fraction of the whole-cell Ca2+ current in most neurons. A common approach to overcome this problem is the use of cellular systems over-expressing the CaV1.3α1 channel protein [31,41,58].

Diverse effects of the auxiliary CaVα2δ-1 subunit have been reported on the properties of cloned high voltage-activated Ca2+ channels. Heterologous co-expression of this protein with neuronal CaV2.2α1 [25,48,59], CaV2.2α2 [27,33], CaV2.3α1 [25,60] or cardiac CaV1.2α1 [19,20,22,25,61,62] and various combinations of CaVβ subunits resulted in a significant increase in current amplitude. The CaVα2δ-1 subunit has been also shown to mediate hyperpolarizing shifts in the voltage dependence of Ca2+ channel activation [25] and inactivation [19,22,25], in addition to regulating the kinetics of current activation [19,62] and inactivation [19,22,59].
Fig. 7. The α2δ-1 auxiliary subunit renders the CaV1.3β1 channels sensitive to gabapentinoids. (A) Average I–V relationships for I\text{Ca} recorded from HEK-293 cells expressing CaV1.3β1/β3 channels, with or without CaV1.2δ-1, in absence and presence of 1 mM gabapentin (GBP) for 48 h. Currents were evoked by 10-mV depolarizing steps from a V\text{h} of −80 mV to potentials between −70 and +50 mV. n = 9–22 recorded cells. (B) Superimposed normalized current traces in absence (control) and presence of the drug. Currents were elicited by a 140 ms depolarizing pulse to −10 mV from a V\text{h} of −80 mV. (C) Comparison of slow and fast components of inactivation (\tau_{\text{inact}}) in absence and presence of GBP. The values of \tau_{\text{inact}} were obtained by fitting the decaying phase of current traces with Eq. (4). The asterisk denotes significant differences (p < 0.05) compared with control. (D) Average I–V relationships for I\text{Ca} recorded from HEK-293 cells expressing CaV1.3β1/β3 channels, with or without CaV1.2δ-1, in absence and presence of 1 mM AdGABA for 48 h. Currents were evoked by 10-mV depolarizing steps from a V\text{h} of −80 mV to potentials between −70 and +50 mV. (E) Superimposed normalized typical current traces in absence (control) and presence of AdGABA. The asterisk denotes significant difference (p < 0.05) compared with control (n = 15–17 cells).

Fig. 8. Inhibition of recombinant N-type CaV channels by gabapentinoids. (A) Mean I\text{Ba} density obtained from HEK-293 cells expressing CaV2.2β1/β3/αδ-1 channels in the control condition and after chronic treatment (48 h) with 1 mM GBP or AdGABA using solutions A and D (Table 1). Currents were elicited by a 140 ms depolarizing pulse to +10 mV from a V\text{h} of −80 mV. n = 7–17 recorded cells. (B) Comparison of the time constants of inactivation (\tau_{\text{inact}}) in absence after the exposure to GBP and AdGABA as indicated. The values of \tau_{\text{inact}} were obtained by fitting the decaying phase of current traces with Eq. (4). The asterisk denotes significant differences (p < 0.05) compared with control.
increase in current amplitude could be attributed to enhanced targeting of expressed CaV1.3 subunits to the plasma membrane, while the effects on the time course and/or voltage dependence of current activation and inactivation suggest a more specific modulation of the channel’s gating.

Very recently, the first successful attempt at knocking out the CaVα2δ-1 subunit has been reported. A comparison of the electrophysiological properties in isolated cardiomyocytes from the CaVα2δ-1 (−/−) and wild-type mice showed that the absence of the CaVα2δ-1 gene results in an attenuated CaV2.3 current amplitude, a decrease in CaV2.3 density, an increase in the time constants (fast and slow), and a depolarizing shift in activation and inactivation [63]. Interestingly, the ablation of the CaVα2δ-1 subunit resulted in reduced GBP binding in the knock-out animals compared with wild-type in either brain or skeletal muscle [63].

Likewise, recent studies have shown that the CaVα2δ-1 subunit might play a more pronounced role in regulating current amplitudes than the other Ca2+ channel auxiliary subunits [25]. In spite of this, to date, there have been relatively few studies showing the effects of CaVα2δ-1 on L-type CaV1.3 channel activity [41, 54]. Indeed, it has not yet been investigated whether the expression of the CaV1.3α1 subunit requires this auxiliary subunit for trafficking to the cell membrane, or for functional expression, or whether CaVα2δ-1 influences the biophysical properties of the CaV1.3 channels in mammalian cells. In the present study we show that co-expression of the CaVα2δ-1 subunit has clear effects on the functional expression of recombinant and native CaV1.3 channels. Over-expression of exogenous CaVα2δ-1 produced a ~3–5-fold increase in the amplitude of currents through recombinant CaV1.3α1/CaVβ2 channels expressed in HEK-293 cells, but had only minor effects on their kinetics or voltage dependence of activation and inactivation. Interestingly, similar effects have been previously reported in Xenopus oocytes in which the amplitude of neuroendocrine CaV1.3α1/CaVβ2 channel currents was increased by ~2–5-fold upon co-expression of the CaVα2δ subunit [41]. This argues either for an effect of the auxiliary subunit on the trafficking of the nascent CaV1.3α1/CaVβ2 channels from the endoplasmic reticulum to the cell membrane, or an effect to stabilize the membrane channels in a functional conformation. Further studies will be necessary to determine whether CaVα2δ-1 affects the properties of the currents acting at the single channel level.

In order to evaluate in more detail the molecular determinants of CaV1.3 channel regulation by the CaVα2δ-1 subunit, a series of site-directed mutants was constructed and functionally analyzed. The amino acids N136 and N184 as well as the sequence between residues R941 to V946 in the protein has been described previously to be important for the subunit-induced current stimulation [27, 33]. The two asparagine residues seem to be glycosylated in vivo while the six amino acids localized between A941 and V946 (Arg-Leu-Leu-Glu-Ala-Val) presumably constitute the proteolytic site in CaVα2δ-1. Substitution of such amino acids renders the CaVα2δ-1 subunit non-functional as shown by patch-clamp experiments in experiments using CaV1.2α1/CaVβ3 channels. Consistent with this, electrophysiological recordings performed in cells expressing mutant constructs indicated that the stimulatory effect of CaVα2δ-1 on macroscopic currents through CaV1.3α1/CaVβ3 channels was partially or completely lost. Last, the same experiments were repeated for a construct in which all cysteine residues in the extracellular region of δ were substituted by methionine or serine (C962M; C984S; C987S; C1032S; C1047S; C1059S). Mutation of the six residues also abolished the stimulatory effect of CaVα2δ-1 on functional CaV1.3α1/CaVβ3 channels, suggesting that the disulphide linkage between the α2 and the δ polyepitides is required for function. Given that the voltage dependence and time course for the activation and inactivation of the channels were practically unaltered, these effects of the CaVα2δ-1 mutant constructs may not be explained by alterations in the functional properties of the channels, but might involve a reduced number of functional channels in the surface of the membrane.

Another interesting finding of our study was that the δ domain of the CaVα2δ subunit exerts an important inhibitory effect on currents through recombinant CaV1.3 channels heterologously expressed in HEK-293 cells. This inhibition was specific given that δ co-expression did not affect endogenous K+ current or heterologously expressed low threshold T-type channels (of the CaV2.2, class), and was not mimicked by the unrelated protein CD8. An exciting issue to be clarified relates to cell pathway(s) by which this inhibition occurs. Based on our findings we could speculate that distinct mechanisms underlie the decrease in current density after δ co-expression. It has been reported that the expression of short variants of the CaV2.2δ1 subunit as well as the over-expression of the neuronal γ2 subunit (stargazing) suppress currents through the activation of an endoplasmic reticulum resident RNA-dependent kinase (PERK) which activates components of the unfolded protein response (UPR) [50, 51]. We therefore tested whether CaV1.3 current suppression by δ involved activation of the UPR using two mutant constructs that have shown to prevent the activation of endogenous PERK [34] and therefore inhibit the UPR. With the PERKΔC and the K618A mutation the suppressive effect of δ remained unaltered, suggesting that activation of PERK may not play a role in the effects of the regulatory subunit. Likewise, to analyze the possible participation of a CaV1.3 channel internalization/degradation-dependent mechanism after δ co-expression, a series of inhibitors was used. We tested chloroquine, filipin III and MG-132, but all internalization inhibitors failed to alter the inhibitory actions of δ as current reduction persisted after treatment. However, several alternative mechanisms could be anticipated to elucidate the δ-induced regulation of CaV1.3 channel functional expression including: (i) that interaction with δ strongly affects the folding of nascent CaVα1.3α1 subunits and affects the interaction with the CaVα2δ and/or CaVβ subunits, (ii) that δ is able to unmask retention signals that prevent the unassembled channel subunits from leaving the ER, and (iii) that δ over-expression could be increasing the degradation rate of the mRNAs for other CaVβ subunit reducing the channel protein levels and hence altering the functional expression of the channels.

Last, the expression and functional integrity of the CaVα2δ-1 subunit in our model system was also confirmed pharmacologically by examining the chronic effects of GBP (1 mM) and AdGABA (1 mM) two anticonvulsant drugs that bind the auxiliary subunit [54, 65]. GBP did not affect the amplitude of the currents and have a minor effect on the voltage dependence of activation. Exposure to the drug did, however, slow down the kinetics of inactivation. These results differ to that reported recently for recombinant neuronal CaV channels in which chronic incubation with GBP (1 mM) reduced currents through CaV2.1α1/CaVβ3/CaVα2δ-2 and CaV2.2α1/CaVβ1/CaVα2δ-1 channels and shifted the voltage dependence of steady-state inactivation to more positive potentials [30]. A possible explanation for this difference is that the affinity of GBP to the CaVα2δ subunit may be modulated by other subunits, and that the effects of the drug depend on the composition and environment of the channel. Likewise, we also found that chronic treatment with AdGABA significantly inhibited macroscopic currents through CaV1.3α1/CaVβ2/CaVα2δ-1 channels. To our knowledge, the inhibitory effect of AdGABA on CaV1.3 channel functional expression, represents a previously uncharacterized action of this drug, and is in agreement with our previous report describing the synthesis and pharmacological profile of AdGABA [54], in which we found that it reduces the functional expression of neuronal recombinant channels of the CaV2.2 class.
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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ceca.2009.08.006.

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Supplementary Fig. S1. Ca²⁺-dependent inactivation and CaV₁.3α₂δ-1 subunit regulation of currents through recombinant CaV₁.3 channels. (A) Representative traces of Ca²⁺ macroscopic current (Iₘacro) recorded from HEK-293 cells expressing CaV₁.3α₁/β₃ channels in absence and presence of α₂δ-1, using solutions B and D (Table 1). Currents were elicited by a 140 ms depolarizing pulse to −10 mV from a Vₜ₉ of −80 mV. (B) Average I–V relationships for Iₘacro recorded from HEK-293 transfected as in A. (C) Voltage dependence of activation of recombinant CaV₁.3α₁/β₃ channels in absence and presence of the CaV₁α₂δ-1 subunit. G–V curves were plotted by converting peak current values of panel B to peak conductance values using Eq. (1), and the mean data were fitted with a Boltzmann function (Eq. (2)). n = 10 cells. (D) Voltage dependence of steady-state inactivation of recombinant CaV₁.3α₁/β₃ channels with or without CaV₁α₂δ-1. Currents were elicited by 2 s conditioning pulses from a Vₜ₉ of −80 mV in 10 mV steps from −110 to +20 mV. Data were plotted against the conditioning pulse, and were fitted with a Boltzmann function (Eq. (3)). n = 14 recorded cells.
Supplementary Fig. S2. The δ subunit inhibits the functional expression of CaV1.3α₁/β₃ channels. (A) Representative traces of $I_{Ca}$ recorded from HEK-293 cells transfected with the CaV1.3α₁/β₃ cDNAs in absence and presence of δ, using solutions B and D (Table 1). Currents were elicited as in A. (B) Average $I$–$V$ relationships for $I_{Ca}$ recorded in cells expressing the CaV1.3α₁/β₃ channels with or without the δ subunit. (C) Relative $I_{Ca}$ densities obtained from cells that expressed the recombinant CaV1.3α₁/β₃ channels alone (control; solid bar), plus the empty vector pcDNA3, the transmembrane protein CD8 or the δ subunit ($n = 10$–$14$ cells). The asterisk denotes a significant difference with respect to the control ($p < 0.05$).
Supplementary Fig. S3. The Ca\textsubscript{\text{v}}\text{\textgreek{d}} domain inhibit current through Ca\textsubscript{\text{v}}2.2/Ca\textsubscript{\text{v}}\text{\beta}_3 channels heterologously expressed in HEK-293 cells. (A) Comparison of $I_{\text{Ba}}$ density in individual HEK-293 cells determined as the current amplitude at $V_m$ of +10 mV normalized by cell capacitance (pA/pF). (B) Representative current traces from control cells ($-\delta$) or cells co-expressing Ca\textsubscript{\text{v}}\text{\textgreek{d}} (+$\delta$) using recording solutions A and D (Table 1). Currents were elicited by voltage steps to +10 mV from a $V_h$ of −80 mV.
Supplementary Fig. S4. The CaVγ subunit is not expressed in the RIN-m5F cells. Aliquots (40 μg) of protein extract from RIN-m5F cells or HEK-293 cells (untransfected or transiently transfected with the CaVγ2 subunit) were electrophoresed on a 10% SDS-polyacrylamide gel and proteins were then transferred to nitrocellulose. Membranes were blocked for 1–2 h in TBST (100 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.5% (v/v) Tween-20) with 5% (w/v) low-fat dried milk and then incubated overnight at 4 °C with a rabbit polyclonal antibody against CaVγ2 subunit. Specific proteins were visualized by using the enhanced chemiluminescence (ECL) Western blotting kit (Amersham Life Science) according to the manufacturer's instructions. As a protein loading control, membranes were striped and incubated with a mouse monoclonal anti-β-actin antibody (lower panel).