Glycosylation of asparagines 136 and 184 is necessary for the $\alpha_2\delta$ subunit-mediated regulation of voltage-gated Ca^{2+} channels

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Received 8 July 2004; revised 23 August 2004; accepted 23 August 2004

Available online 11 September 2004

Edited by Maurice Montal

Abstract The $Ca_V\alpha_2\delta$ auxiliary subunit is a glycosylated protein that regulates the trafficking and function of voltage-gated Ca^{2+} channels. One of the most prominent roles of $Ca_V\alpha_2\delta$ is to increase whole-cell Ca^{2+} current amplitude. Using N-glycosidase F and truncated forms of $Ca_V\alpha_2\delta$, earlier studies suggested an important role for N-linked glycosylation in current stimulation. Here, we used site-directed mutagenesis and heterologous expression in HEK-293 cells to examine the impact of individual glycosylation sites within the $Ca_V\alpha_2\delta$ subunit on the regulation of Ba^{2+} currents through recombinant Ca^{2+} channels. We found two N-glycosylation consensus sites (NX(S/T)) in the extracellular α_2 domain of the protein that are functional. Substitution of asparagines for glutamines at amino acid positions 136 and 184 rendered these sites non-functional as shown by patch-clamp experiments. These results corroborate that N-glycosylation is required for the $Ca_V\alpha_2\delta$ subunit-induced current stimulation and suggest that sites N136 and N184 are directly involved in this action. Likewise, N136Q and N184Q mutations prevented whole-cell current stimulation without altering its kinetic properties, suggesting a regulation on the number of functional channels at the plasma membrane.

Keywords: $\alpha_2\delta$ subunit; Ca^{2+} channel; $Ca_V2.2$ channel; *N*-glycosylation; HEK-293 cell

1. Introduction

Voltage-gated Ca^{2+} (Ca_V) channels are membrane-spanning proteins that mediate Ca^{2+} entry into all excitable and many non-excitable cells, thereby initiating a number of important cellular processes including gene transcription, muscle contraction, and neurotransmitter release. Ca_V channels are functionally diverse and five different types have thus far been defined: T, L, N, P/Q and R [1]. These channel types can be separated into two classes based on threshold of voltage activation: low voltage- and high voltage-activated channels (LVA and HVA, respectively). HVA channels are oligomeric complexes consisting of one pore-forming (α_1) subunit and three auxiliary subunits: $\alpha_2\delta$, β, and γ [2,3]. According to molecular studies, the $Ca_V2.2$ (formerly α_{1B}) subunit originate the N-type Ca^{2+} channels present in presynaptic terminals whose activation is essential in excitation–secretion coupling [2,4]. Expression of multiple $Ca_V2.2$ splice variants [5,6] and association with auxiliary subunits [7,8] determine the diversity of N-type Ca^{2+} currents in the nervous system.

The auxiliary $Ca_V\alpha_2\delta$ subunit is a transmembrane protein that can be encoded by four different genes with multiple splice variants possible for each gene [9]. The $Ca_V\alpha_2\delta$ protein is post-translationally cleaved into a long, N-terminal, extracellular α_2 polypeptide and a shorter, membrane-anchored δ protein, which remain covalently linked by disulfide bonds [10,11]. The ultimate functional properties of the Ca_V channel complex can be finely tuned by the presence of $Ca_V\alpha_2\delta$ [9,12]. Upon recombinant co-expression, $Ca_V\alpha_2\delta$ regulate channel activity by enhancing the whole-cell current density [13–19]; as well as by shifting the voltage dependence of activation to more negative potentials [19,20], and/or increasing the steady-state inactivation of the ionic [17,20] and gating [21] currents. In addition, $Ca_V\alpha_2\delta$ possess binding sites for the antiepileptic drug gabapentin [22–24], which exerts acute [25–27] and chronic inhibitory actions on Ca_V channel functional expression [28,29]. Lastly, the mouse mutants *ducky*, *ducky*^{2J} [30–32] and *entla* [33] are shown to be functional $Ca_V\alpha_2\delta$ null alleles. Homozygote animals exhibit decreased Ca_V currents in cerebellar neurons and develop ataxia, absence epilepsy and paroxysmal dyskinesia [33,34].

Experimental evidence suggests that one of the most prominent and potentially important roles of the $Ca_V\alpha_2\delta$ subunit is to enhance Ca^{2+} current amplitude [9,12]. However, the determinants in $Ca_V\alpha_2\delta$ responsible for the up-regulation in channel activity have not been clearly identified or separated, though this process has been attributed to glycosylation. It has been demonstrated that deglycosylation with PNGase F of intact *Xenopus* oocytes expressing recombinant $Ca_V2.1/\beta_4/\alpha_2\delta$-1 channels resulted in a significant reduction of macroscopic current amplitude [16]. In contrast, identical treatment of oocytes expressing only $Ca_V2.1/\beta_4$ subunits did not have an effect on current amplitude [16]. Likewise, N-terminal truncations of $Ca_V\alpha_2\delta$ resulted in reductions in current amplitude through $Ca_V2.1/\beta_4/\alpha_2\delta$-1 channels expressed in oocytes, presumably by a gradual deletion of potential *N*-glycosylation sites [16]. The smallest N-terminal truncation tested (NΔ28–184) completely abolished the functional activity of the $Ca_V\alpha_2\delta$-1 subunit [16]. However, to date there has been no systematic approach toward delineating the structural determinants that are responsible for the effects mediated by $Ca_V\alpha_2\delta$. In the current report,

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0014-5793/$22.00 © 2004 Published by Elsevier B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/j.febslet.2004.08.054

we show the identification of two *N*-linked glycosylation sites (N136 and N184) that are critical for the $Ca_V\alpha_2\delta$ subunit-mediated regulation of neuronal Ca_V channels.

2. Materials and methods

2.1. Site-directed mutagenesis

The cDNA coding the $Ca_V\alpha_2\delta$-1 was subcloned into the *Sac*II and *Eco*RI sites of pIRES-hrGFP-1a vector (Stratagene). The vector contains a CMV promotor, a multiple cloning site, followed by an internal ribosomal entry site (IRES) from encephalomyocarditis virus, then the gene encoding the Green Fluorescent Protein (GFP) and poly A region (SV40). N → Q mutations at two amino acid residues from the N-terminus (at positions 136 and 184) of the $Ca_V\alpha_2\delta$-1 subunit were introduced to prevent *N*-linked glycosylation. These point mutations were introduced with ~40-mer synthetic oligonucleotides using the Quick-Change XL-mutagenesis kit (Stratagene). cDNAs of the mutant channel subunits were sequenced in both directions on an automated sequencer (ABIPrism310, Perkin–Elmer Applied Biosystems).

2.2. SDS–PAGE and immunoblot analysis

Microsomes from transfected HEK-293 cells were prepared as previously described [17,38]. Samples were subjected to gel electrophoresis under reducing conditions using 2.5% (v/v) β-mercaptoethanol. All samples were heated at 90 °C for 5 min and 200 μg of protein/slot were loaded on 5% polyacrylamide gels. For Western blot analysis, proteins were blotted onto nitrocellulose membranes and blots were developed as described [17,38] with specific polyclonal primary antibodies (Rabbit 136) [16] used in a 1:4000 dilution. Rabbit 136 antibody recognizes a 17 amino acid sequence (residues 839–856) of the rat $\alpha_2\delta$-1 subunit. The specific protein bands were detected using the ECL detection method according to the manufacturers' instructions.

2.3. Cell culture and recombinant $Ca_V2.2$ channel expression

Human embryonic kidney (HEK-293) cells were grown in DMEM-high glucose supplemented with 10% equine serum, 2 mM L-glutamine, 110 mg/l sodium pyruvate and 50 μg/ml gentamycin, at 37 °C in a 5% CO_2–95% air humidified atmosphere. After splitting the previous day and seeding at ~60% confluency, cells were transfected using the Lipofectamine Plus reagent (Gibco BRL) with 1.2 μg plasmid cDNA encoding the rabbit brain N-type Ca^{2+} channel $Ca_V2.2$ pore-forming subunit (formerly α_{1B}; GenBank accession number D14157) [35] in combination with 1.2 μg cDNA of the rat brain $Ca_V\beta_3$ (M88751) [36], and 1.2 μg cDNA coding the rat brain $Ca_V\alpha_2\delta$-1 (M86621) [37] or its mutants.

2.4. Electrophysiology

Forty eight hours after transfection, cells expressing the GFP reporter gene were subjected to the whole-cell mode of the patch clamp technique [39]. Briefly, currents were recorded with an Axopatch 200B amplifier (Axon Instruments) and acquired on line using a Digidata 1320A interface with pClamp8 software (Axon Instruments). After establishing the whole-cell mode, capacitive transients were canceled with the amplifier. Currents were obtained from a holding potential (HP) of −80 mV applying test pulses every 20 s. 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 (C_m) was determined as described previously [40] and used to normalize currents. The bath recording solution contained (in mM) 10 $BaCl_2$, 125 TEA-Cl, 10 HEPES and 15 glucose (pH 7.3). The internal solution consisted of (in mM) 110 CsCl, 5 $MgCl_2$, 10 EGTA, 10 HEPES, 4 Na-ATP and 0.1 GTP (pH 7.3). Experiments were performed at room temperature (~25 °C).

2.5. Data analysis

The data are given as means ± S.E. Statistical differences between two means were determined by Student's *t* tests ($P < 0.05$). Current activation and decay were fitted with single exponential equations of the form: $A\exp(-t/\tau) + c$, where A is the initial amplitude (pA), t is time (ms), τ is the time constant and c is a constant. Steady-state inactivation curves were fitted with a Boltzmann function: $I_{Ba} = I_{max}/(1+\exp[(V_m - V_{1/2})/k])$, where the current amplitude I_{Ba} has decreased to a half-amplitude at $V_{1/2}$ with an *e*-fold change over k mV.

3. Results

We first searched for potential glycosylation consensus sites (NX(S/T)) in the sequence of the rat brain $Ca_V\alpha_2\delta$-1 subunit. By using the NetNGlyc1.0 software [41] (available at the URL http://www.cbs.dtu.dk/services/NetNGlyc/), we detected 10 putative *N*-linked glycosylation sites in the extracellular α_2 domain. Interestingly, two of them were present in the 28–184 stretch (Fig. 1A). Next, to test whether these sites were post-translationally modified and whether such modifications would entail any functional consequences, we created two single and one double *N*-glycosylation mutants (N136Q and/or N184Q). All cDNA constructs were analyzed by *Xho*I digestion (Fig. 1B) and sequenced to confirm the desired mutations.

The wild-type $Ca_V\alpha_2\delta$-1 and its three mutated versions were expressed in HEK-293 cells and analyzed by immunoblotting. As shown in Fig. 1C, when membranes of wild-type $Ca_V\alpha_2\delta$-1 expressing cells were resolved by SDS–PAGE and probed with a polyclonal anti-α_2 antibody, a specific band of ~130 kDa was detected. In contrast, Western blot of the $Ca_V\alpha_2\delta$-1 single glycosylation mutants showed shifts in the mobility of the ~130 kDa protein to ~126 kDa on a 5% gel, suggesting impaired *N*-glycosylation of sites N136 and N184. In addition, evidence of a lower molecular weight protein corresponding to the unglycosylated $Ca_V\alpha_2\delta$-1 double mutant (DM) was also obtained. These results strongly suggest that sites N136 and N184 are glycosylated in vivo.

In order to evaluate the functional role of *N*-linked carbohydrate modification of the $Ca_V\alpha_2\delta$-1 subunit, the whole-cell patch clamp technique was used to study the macroscopic Ba^{2+} currents (I_{Ba}) through recombinant Ca_V channels ($Ca_V2.2/\beta3$) in HEK-293 cells transiently expressing wild-type $Ca_V\alpha_2\delta$-1 and its mutants. Fig. 2A shows representative current traces recorded during depolarizing voltage steps to +10 mV from a HP of −80 mV. Control experiments carried out using cells transfected with the wild-type $Ca_V\alpha_2\delta$-1 showed that the current levels ranged up to −2.8 nA, with an average peak current of -782 ± 96 pA (Fig. 2B). Recordings performed in cells transfected with the single mutations (N136Q and N184Q) indicated that the stimulatory effect of the auxiliary subunit was partially lost (N136Q, -451 ± 83 pA; N184Q, -439 ± 94 pA). Both mutants prevented equally the $Ca_V\alpha_2\delta$-mediated regulation of Ca_v channels. Interestingly, it was found that the evident current stimulation observed in wild-type transfected cells was absent in the cells expressing the $Ca_V\alpha_2\delta$-1 subunit harboring the double mutation (DM, -242 ± 49 pA; $-\alpha_2\delta$, -198 ± 67 pA). These data are further illustrated in Fig. 3A, which shows the I_{Ba} density (current amplitudes divided by the respective values of C_m) as a function of the voltage step in cells transfected with the wild-type $Ca_V\alpha_2\delta$-1 and its mutations. These current density–voltage relationships indicate that I_{Ba} is activated at potentials positive to −20 mV and reach its peak at potentials close to +10 mV. Notably, the effects of the $Ca_V\alpha_2\delta$-1 mutant subunits were observed at almost all potentials explored.

We next investigated whether the effects of the mutations could be explained by alterations in the macroscopic kinetic properties. Normalized currents obtained from either control

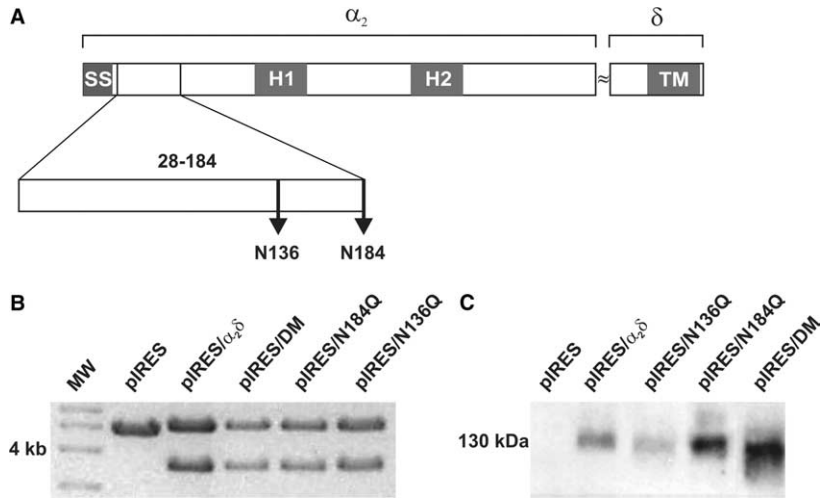

Fig. 1. Mutation of two NX(S/T) motifs to delete extracellular *N*-linked glycosylation sites in the $Ca_V\alpha_2\delta$ subunit. (A) Schematic representation of the $Ca_V\alpha_2\delta$-1 subunit. SS denotes signal sequence, H1 and H2 indicate hydrophobic regions; the protein is represented as a horizontal rectangle, and ≈ indicates the cleavage site between the α_2 and δ peptides. TM denotes the transmembrane domain in δ. Arrows indicate the positions of the two asparagine residues (N136 and N184) in the 28–184 stretch accessible for glycosylation. (B) Agarose gel analysis of the cDNA constructs coding the full-length $Ca_V\alpha_2\delta$-1 (pIRES/$\alpha_2\delta$) and its *N*-glycosylation mutants (pIRES/N136Q, pIRES/N184Q and the double mutation pIRES/DM). Restriction analysis showed the expected ~5.4 and ~3.5 Kb fragments corresponding to the pIRES-hrGFP-1a vector and the $Ca_V\alpha_2\delta$-1 inserts (wild-type and mutants). (C) Western blot analysis of membranes from mock transfected HEK-293 cells (pIRES) or cells expressing the wild-type (pIRES/$\alpha_2\delta$), and the *N*-glycosylation mutants of the $Ca_V\alpha_2\delta$-1 subunit (pIRES/N136Q, pIRES/N184Q and pIRES/DM).

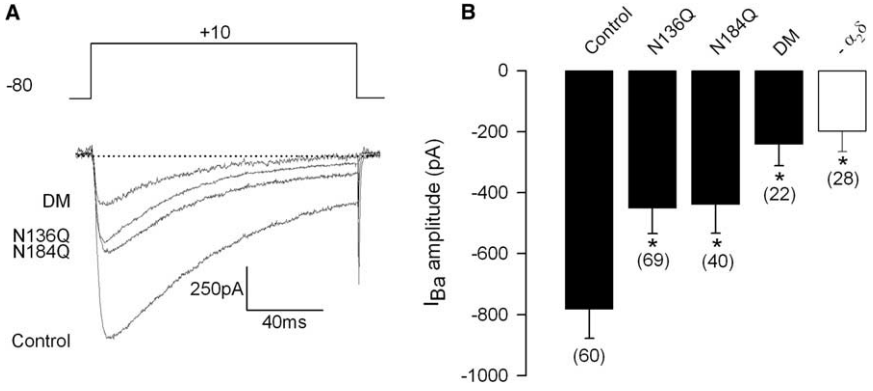

Fig. 2. Mutations at sites N136 and N184 in the extracellular α_2 domain disrupt the function of $Ca_V\alpha_2\delta$-1. (A) Superimposed representative whole-cell Ba^{2+} current traces through recombinant Ca_V channels ($Ca_V2.2/\beta_3$) in HEK-293 cells expressing wild-type (control) and *N*-glycosylation mutants of the $\alpha_2\delta$-1 subunit (N136Q, N184Q and DM). Currents were generated by applying 140 ms activating pulses at +10 mV from a HP of −80 mV. (B) Summary histograms showing the average peak current from control and cells expressing the $Ca_V\alpha_2\delta$-1 *N*-glycosylation mutants. The open bar shows the average current amplitude in the absence of the $\alpha_2\delta$ subunit. The number of recorded cells is indicated in parentheses, and the asterisks denote significant differences ($P < 0.05$).

or cells expressing $Ca_V\alpha_2\delta$-1 mutant subunits showed that the temporal course of the current traces was very similar, suggesting that the activation and inactivation rate of the channels was not altered (data not shown). Accordingly, neither the time to peak nor the time constant for the activation of the current (τ_{act}) were significantly modified (Fig. 3B; Table 1). Likewise, we generated complete voltage-dependent inactivation curves for the recombinant Ca_V channels expressed. Except for the marked reduction in the maximal current amplitude observed when the mutant subunits were present (~2-fold; N136Q and N184Q, and ~4-fold for DM; see Fig. 2B), the other Boltzmann parameters describing the inactivation curves (i.e. $V_{1/2}$ and k) were virtually the same to the corresponding values obtained from cells expressing wild-type $Ca_V\alpha_2\delta$-1 (Fig. 4A; Table 1). Lastly, the time constant for the inactivation of the current (τ_{inact}) and the percentage of current remaining after 140 ms activating pulses were practically indistinguishable between control and cells expressing the mutant subunits (Fig. 4B; Table 1). Thus, alterations in the activation and/or inactivation properties of the channels may not be contributing to the observed reductions in the magnitude of the whole-cell currents.

4. Discussion

To uncover potential molecular determinants for the $Ca_V\alpha_2\delta$ subunit-mediated up-regulation of Ca^{2+} channels, in

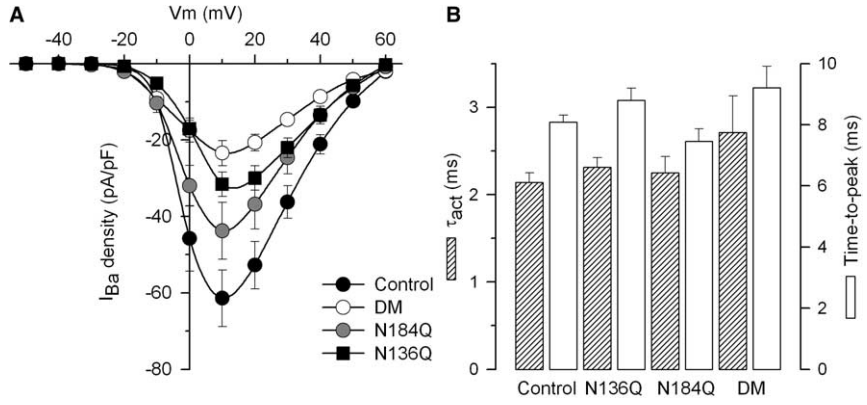

Fig. 3. Glycosylation at sites N136 and N184 of $Ca_V\alpha_2\delta$-1 influences Ca^{2+} channel functional expression without affecting activation. (A) Current–voltage relationship for cells transfected with $Ca_V2.2/\beta_3$ and wild-type (control) and *N*-glycosylation mutants of $\alpha_2\delta$-1: N136Q (black squares) or N184Q (grey circles) or the double mutation, DM (white circles). Data points represent the average current density ± S.E.M. at a test potential as listed, from 6 to 15 cells recorded in each group. (B) Average I_{Ba} time to peak and time constants (τ_{act}) for the activation of the recombinant Ca_V channels. The values of τ_{act} were estimated by fitting the rising phase of the current traces with an exponential function (see Section 2). Average ±S.E.M. from 10 to 45 cells in each group is shown.

Table 1
Whole-cell current parameters in HEK-293 cells transfected with $Ca_V2.2/\beta_3$ and the wild-type or the *N*-linked glycosylation mutants of the $\alpha_2\delta$-1 auxiliary subunit

$\alpha_2\delta$-1 construct	Activation		Inactivation			
	Time to peak (ms)[a]	τ (ms)[a]	τ (ms)[a]	*I* remaining (%)[a]	$V_{1/2}$ (mV)	*k* (mV)
WT	8.1 ± 0.2	2.1 ± 0.1	69.5 ± 3.8	21.6 ± 1.5	−51.0	−11.1
N136Q	8.8 ± 0.4	2.3 ± 0.1	68.7 ± 4.0	23.8 ± 1.9	−51.6	−13.5
N184Q	7.5 ± 0.4	2.2 ± 0.2	63.4 ± 6.9	20.0 ± 2.9	−54.5	−11.8
DM	9.2 ± 0.7	2.7 ± 0.4	77.8 ± 16.2	22.7 ± 2.7	−52.3	−13.5
$-\alpha_2\delta$	9.4 ± 0.7	2.2 ± 0.2	113.0 ± 31.7	36.6 ± 5.7	−51.2	−13.5

Each value is mean ± S.E.M.
WT: wild-type; DM: double mutation.
[a] Currents were recorded during depolarizations from −80 to +10 mV.

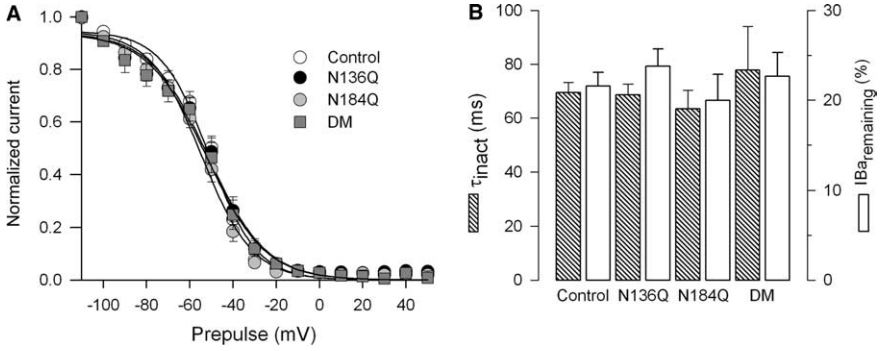

Fig. 4. Glycosylation at sites N136 and N184 of $Ca_V\alpha_2\delta$-1 does not affect Ca^{2+} channel inactivation. (A) Normalized, averaged steady-state inactivation curves for $Ca_V2.2/\beta_3$ recombinant channels co-expressing wild-type (control) and the *N*-glycosylation mutants. Symbols denote average ±S.E.M. of the normalized data at a given membrane potential for 7–18 different cells. Solid lines represent single Boltzmann fits to the averaged data. (B) Average time constants of inactivation (τ_{inact}) and percentage of current remaining 140 ms into the depolarizing pulse in cells expressing the wild-type (control) and *N*-glycosylation mutants of $Ca_V\alpha_2\delta$-1. The values of τ_{inact} were obtained by fitting the decaying phase of the currents with an exponential function (see Section 2). Average ±S.E.M. from 9 to 35 recorded cells in each group is shown.

this study we characterized the importance of two *N*-linked glycosylation sites (N136 and N184) located at the extracellular α_2 domain of the protein. Our findings show that $Ca_V\alpha_2\delta$-1 heterologously expressed in the HEK-293 cells could be glycosylated in sites N136 and N184, and by mutagenesis of both residues we were able to create non-functional $Ca_V\alpha_2\delta$-1 glycosylated deficient variants. Consistent with the notion that the *N*-glycosylation of the $Ca_V\alpha_2\delta$-1 subunit is responsible for an important stimulation of channel activity [16], co-expression of wild-type $Ca_V\alpha_2\delta$-1 with $Ca_V2.2$ and $Ca_V\beta_3$ subunits resulted in a 4-fold increase in whole-cell Ca^{2+} current amplitude. To determine whether

both or only one glycosylation site on $Ca_V\alpha_2\delta$ needs to be glycosylated to increase current amplitude, we tested single glycosylation mutants. When either the N136Q or the N184Q mutant was co-expressed with $Ca_V2.2/Ca_V\beta_3$ channels, the current was reduced to ~56% of control. These results indicate that both *N*-glycosylation sites are critical for the $Ca_V\alpha_2\delta$-mediated regulation of Ca^{2+} channels. In addition, when the double glycosylation mutant was co-transfected, the current was further reduced (to ~31% of control). Interestingly, the average current amplitude in the presence of the DM of glycosylation was very similar to the magnitude of the currents in the absence of the $\alpha_2\delta$ subunit (Fig. 2B). However, the waveforms in the two conditions were different. Currents in the absence of any $\alpha_2\delta$ subunits inactivate significantly slower (see τ of inactivation and *I* remaining in Table 1), suggesting that all the abnormally glycosylated proteins, including the DM still interact with the channel.

Taken as a whole, our data suggest that the less glycosylated $Ca_V\alpha_2\delta$-1 subunits are inefficient in trafficking to the cell membrane or are less stable once they reach the membrane [16]. In keeping with this view, the functional properties of the channels were practically unaltered (Figs. 3B and 4) and only major alterations were observed in the maximal conductance which depends on the number of functional channels. Thus, conceivable alterations in the number of Ca_V channels at the plasma membrane might be accounting for the effects of the $Ca_V\alpha_2\delta$-1 *N*-glycosylation mutants.

Indeed, glycosylation of some ion channels has been shown to influence their membrane targeting and surface expression [42–44]. In particular, a number of K^+ channel types are glycosylated on asparagine residues and mutation of these residues indicates that glycosylation may drastically increase the stability and cell surface expression of the channel proteins [44–48]. It is well known that in the absence of *N*-linked glycosylation, protein may be retained in the endoplasmic reticulum (ER) and degraded [45,47]. Interestingly, in HEK-293 cells, glycosylation stabilizes the Shaker K^+ channel and drastically enhances its trafficking to the cell surface probably by an interaction with calnexin and/or calreticulin which protects newly made Shaker proteins from retrotranslocation and degradation [49,50]. However, interactions between the $Ca_V\alpha_2\delta$-1 auxiliary subunit and the ER resident proteins have not been documented yet. Alternatively, the wild-type and unglycosylated $Ca_V\alpha_2\delta$-1 proteins may be differentially sensitive to ER-associated retention because are transported out of the ER at different rates. In this scenario, glycosylation may increase the rate of folding and assembly of the $Ca_V\alpha_2\delta$-1 protein, thereby allowing a more rapid exit from the ER. Exit on time from the ER may be a key factor in preventing the wild-type $Ca_V\alpha_2\delta$-1 but not the unglycosylated mutant proteins from retention. Hence, retention of $Ca_V\alpha_2\delta$-1 glycosylation mutants in the ER, and the kinetics of ER exit of the wild-type and unglycosylated $\alpha_2\delta$ proteins are interesting topics for future studies.

Acknowledgements: This work was supported by grants from Conacyt and The Miguel Aleman Foundation (to R.F.). Doctoral (A.S., A.A.) and postdoctoral (N.O.) fellowships from Conacyt are acknowledged. We thank L. Sánchez for expert technical assistance and G. Aguilar for assistance with DNA sequencing. We are in debt with Drs. B.A. Adams (Utah State University) and K.P. Campbell (University of Iowa) for their generous gift of the cDNA clones and antibodies.

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