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**Proteolytic cleavage of the voltage-gated Ca<sup>2+</sup> channel  $\alpha_2\delta$  subunit:  
structural and functional features**

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## **Abstract**

By mediating depolarization-induced  $\text{Ca}^{2+}$  influx high voltage-activated (HVA)  $\text{Ca}^{2+}$  channels control a variety of cellular events. These heteromultimeric proteins are composed of an ion-conducting ( $\alpha_1$ ) and three auxiliary ( $\alpha_2\delta$ ,  $\beta$  and  $\gamma$ ) subunits. The  $\alpha_2\delta$  subunit enhances the trafficking of the channel complex to the cell surface and increases channel open probability. To exert these effects,  $\alpha_2\delta$  must undergo important post-translational modifications including a proteolytic cleavage that separates the extracellular  $\alpha_2$  from its transmembrane  $\delta$  domain. After this proteolysis both domains remain linked by disulfide bonds. In spite of its central role in determining the final conformation of the fully mature  $\alpha_2\delta$ , almost nothing is known about the physiological implications of this structural modification. In the current report, by using site-directed mutagenesis, the proteolytic site of  $\alpha_2\delta$  was mapped to amino acid residues Arg-941 and Val-946. Substitution of these residues renders the protein insensitive to proteolytic cleavage as evidenced by the lack of molecular weight shift upon treatment with a disulfide reducing agent. Interestingly, these mutations significantly decreased whole-cell patch clamp currents without affecting the voltage-dependence or kinetics of the channels, suggesting a reduction in the number of channels targeted to the plasma membrane.

## Introduction

Ca<sup>2+</sup> entry through voltage-activated Ca<sup>2+</sup> (Ca<sub>v</sub>) channels contributes to many physiological processes in the nervous system, including neurotransmitter release, neuronal excitability and plasticity, gene expression, neurite outgrowth, synaptogenesis and neuronal survival and differentiation. Ca<sub>v</sub> channels have been subdivided according to their electrophysiological properties into: i) low voltage-activated (LVA or T-type) channels, and ii) high-voltage activated (HVA) channels, a class that includes the L-, N-, P/Q-, and R-types (Catterall *et al.*, 2005; Lacinova, 2005). Molecular studies indicate that Ca<sub>v</sub> channels of the HVA class are oligomeric complexes consisting of an ion-conducting  $\alpha_1$  subunit and auxiliary  $\alpha_2\delta$ ,  $\beta$  and  $\gamma$  subunits (Felix, 2005; Lacinova, 2005). The auxiliary subunits modulate the trafficking and the biophysical properties of the  $\alpha_1$  subunit (Arikkath and Campbell, 2003; Lacinova, 2005).

The  $\alpha_2\delta$  subunit is a transmembrane protein complex that is encoded by a single gene. Since the identification of the first  $\alpha_2\delta$  subunit (Ellis *et al.*, 1988), four genetically distinct  $\alpha_2\delta$  subunits have been described (Klugbauer *et al.*, 1999; Qin *et al.*, 2002). Each one of these proteins is differentially expressed in various tissues, including skeletal muscle, heart and brain. At the protein level, all four  $\alpha_2\delta$  subunits show conserved glycosylation sites, cysteine residues and predicted hydrophobicity profiles (Arikkath and Campbell, 2003; Klugbauer *et al.*, 2003). It has been shown that heterologous expression of each of these  $\alpha_2\delta$  subunits induces an enhancement in the ionic current produced by the  $\alpha_1$  subunit expression, associated with an increase in the number of ligand binding sites for the channel (Felix, 1999; Arikkath and Campbell, 2003; Klugbauer *et al.*, 2003). These data indicate that  $\alpha_2\delta$  produces an increase in cell surface expression of Ca<sup>2+</sup> channels. Likewise, functional studies have revealed that the  $\alpha_2\delta$  subunits cause increases in current amplitude, faster activation and inactivation kinetics and

hyperpolarizing shifts in the voltage dependence of the expressed currents (Singer *et al.*, 1991; Welling *et al.*, 1993; Shistik *et al.*, 1995; Gurnett *et al.*, 1996; Bangalore *et al.*, 1996; Felix *et al.*, 1997; Jones *et al.*, 1998; Qin *et al.*, 1998; Shirokov *et al.*, 1998; Klugbauer *et al.*, 1999; Hobom *et al.*, 2000; Sandoval *et al.*, 2004; Yasuda *et al.*, 2004; Canti *et al.*, 2005; Obermair *et al.*, 2005).

Molecular studies have established that the  $\alpha_2$  domain of the protein is extensively glycosylated, a post-translational modification that is essential in maintaining the stability of the interaction with the  $\alpha_1$  subunit (Gurnett *et al.*, 1996; 1997). It is also a major determinant in the ability of the protein to stimulate current amplitude (Gurnett *et al.*, 1996; Sandoval *et al.*, 2004). Likewise, it has been documented that the  $\alpha_2\delta$  subunit is translated as a precursor polypeptide that is post-translationally cleaved (De Jongh *et al.*, 1990; Jay *et al.*, 1991). The  $\delta$  domain, which contains a single transmembrane segment, maintains the  $\alpha_2$  domain close to the plasma membrane. Despite proteolytic cleavage, the linkage of both domains is maintained by disulfide bridges formed between cysteine residues found in both proteins (Felix, 1999; Klugbauer *et al.*, 2003; Lacinova, 2005). The mechanisms that underlie the proteolytic cleavage and the disulfide linkage still remain unclear.

In the present report, we used site-directed mutagenesis and heterologous expression in HEK-293 cells to localize the putative site of proteolysis within  $\alpha_2\delta$ , and examine the impact of the proteolytic processing of this auxiliary subunit on the functional activity of neuronal recombinant  $\text{Ca}_v$  channels.

## Materials and methods

*Site-directed mutagenesis.* Amino acids 941 and 946 were substituted by site directed mutagenesis of the corresponding cDNA to prevent proteolytic cleavage of the  $\alpha_2\delta$ -1b subunit. To this end, we used the recombinant bicistronic expression plasmid PIRE5/ $\alpha_2\delta$  (Sandoval *et al.*, 2004), which carried the entire protein-coding region for the rat brain  $\alpha_2\delta$ -1b  $\text{Ca}^{2+}$  channel auxiliary subunit (GenBank accession number M86621) and for the green fluorescent protein (GFP) coupled by an internal ribosomal entry site (IRES) sequence. The point mutations were introduced with ~40-mer synthetic oligonucleotides using the Quik-Change XL-mutagenesis kit (Stratagene, La Jolla, CA, USA). Initially, a single amino acid mutation changing a glutamic acid to a glutamine residue (E944Q) was created. Next, a quadruple mutation (P4) was done on the single cDNA mutant E944Q and lastly, a sextuple mutation (P6) was obtained on the P4 cDNA mutant by using suitable mutagenic primers (Fig. 1A). cDNAs of all three mutant channel subunits were sequenced on an automated sequencer (ABIPrism310, Perkin-Elmer Applied Biosystems, Foster City, CA, USA).

*Cell culture and recombinant  $\text{Ca}_V$  channel expression.* Human embryonic kidney (HEK-293) cells were maintained in DMEM-high glucose supplemented with 10% equine serum, 1% L-glutamine, 110 mg/l sodium pyruvate and antibiotics, at 37°C in a 5%  $\text{CO}_2$ -95% air humidified atmosphere. Gene transfer was performed using Lipofectamine Plus reagent (Invitrogen, Carlsbad, CA, USA). Briefly, for a 35 mm Petri dish of HEK-293 cells, 2  $\mu\text{g}$  of the plasmid cDNA encoding the rabbit brain N-type  $\text{Ca}^{2+}$  channel  $\text{Ca}_V2.2$  pore-forming subunit (D14157; Fujita *et al.*, 1993) in combination with 2  $\mu\text{g}$  cDNA of the rat brain  $\beta_3$  (M88751; Castellano *et al.*, 1993) and 2  $\mu\text{g}$  cDNA coding the wild-type rat brain  $\alpha_2\delta$ -1 or its mutants, were premixed with 6  $\mu\text{l}$  of Lipofectamine in 100  $\mu\text{l}$  serum-free medium according to the manufacturer's instructions.

The solution was then added to the dish and cells grown at 37°C for 24 h, when medium was changed. Cells were then harvested at 48 h.

*SDS-PAGE and Western blotting.* Microsomes from transfected HEK-293 cells were prepared as previously described (Felix *et al.*, 1997; Gurnett *et al.*, 1997). Samples were subjected to gel electrophoresis under denaturing conditions. All samples were heated at 95°C for 5 min, and 100 µg of protein/slot were loaded on 5% polyacrylamide gels. For Western blot analysis, proteins were transferred onto nitrocellulose membranes, and blots were developed as described (Felix *et al.*, 1997; Gurnett *et al.*, 1997) with specific Ca<sub>v</sub>α<sub>2</sub> polyclonal primary antibodies (Rabbit 136; Gurnett *et al.*, 1996) used in a 1:4000 dilution. The secondary antibody was a goat anti-rabbit IgG horseradish peroxidase (Zymed Invitrogen, Carlsbad, CA, USA) used at a dilution of 1:6000. The specific protein bands were detected using the enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions.

*Electrophysiological recording.* Ionic currents from HEK-293 cells were recorded using the whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981) 48 h after transfection. Ba<sup>2+</sup> was used as the charge carrier. The extracellular solution contained (in mM): 10 BaCl<sub>2</sub>, 125 TEA-Cl, 10 HEPES and 10 glucose (pH 7.3). The intracellular solution contained (in mM): 110 CsCl, 5 MgCl<sub>2</sub>, 10 EGTA, 10 HEPES, 4 Na-ATP and 0.1 GTP (pH 7.3). Recordings were made with an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) and acquired on-line using a Digidata 1320A interface with pClamp8 software (Molecular Devices). The offset potential between the pipette and bath solutions was zeroed prior to seal formation. Capacitive transients were canceled with the amplifier. Series resistance values were typically 2-10 MΩ, and no records were used in which the voltage error (as defined by  $V_{er} = I_{max}$

$\times R_a$ ) was greater than 5 mV. 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 (Avila *et al.*, 2004) and used to normalize currents. Curve fitting and statistical analysis were carried out using the SigmaPlot 8.0 software package (SPSS Inc., Chicago, IL, USA). The significance of observed differences was evaluated by nonpaired Student's *t* test.  $P < 0.05$  was considered to be significant.



## Results

Before investigating the functional relevance of the proteolytic cleavage of the  $\alpha_2\delta$  auxiliary subunit on neuronal recombinant  $\text{Ca}_v$  channel activity, it was necessary to delimit the putative proteolytic site within the protein. Initial N-terminal sequencing studies on the rabbit skeletal muscle  $\text{Ca}_v$  channel  $\delta$  subunit suggested that cleavage of the  $\alpha_2\delta$ -1 precursor occurs between residues Glu-934 and Ala-935 (De Jongh *et al.*, 1990). Sequence alignment of this deduced proteolytic site with the rat brain  $\alpha_2\delta$ -1b retrieved from the GenBank corroborated the presence of these residues at positions 944 and 945, respectively. Given that many deduced sites of proteolysis are of 6 amino acid residues length, and the cleavage site occurs after the fourth amino acid we speculated that the proteolytic site of the rat brain  $\alpha_2\delta$ -1b used in this study might be positioned between Arg-941 and Val-946 (Fig. 1A).

To test whether this site is indeed post-translationally modified and whether such a modification entails any functional consequence, we produced one single and two multiple mutants of the rat brain  $\alpha_2\delta$ -1b (Fig. 1A; see Material and Methods for details). All cDNA constructs were sequenced to confirm the desired mutations. The wild-type  $\alpha_2\delta$  and its mutated versions were then expressed in HEK-293 cells and analyzed by immunoblotting. This analysis showed that under non-reducing conditions the wild-type protein and the  $\alpha_2\delta$  subunits harboring the single (E944Q) and the quadruple (P4) mutations migrate as high molecular mass proteins of about 150 kDa. Upon reduction, the majority of these proteins migrated as products of the predicted size for the  $\alpha_2$  peptide (~130 kDa). This result suggested that the wild-type and the mutant proteins E944Q and P4 were proteolytically cleaved into separate  $\alpha_2$  and  $\delta$  peptides (Fig. 1B). Expression of the protein that had all six amino acids of the putative proteolytic site mutated (P6) also resulted in the presence of a high molecular mass species (~150 kDa) under non-

reducing conditions which was expected. However upon reduction, the size of this high molecular weight band remained unchanged, indicating that the P6 protein is not susceptible to proteolysis (Fig. 1B).

In order to evaluate the functional role of the proteolytical processing of the  $\alpha_2\delta$  subunit, the patch clamp technique was used to study macroscopic  $\text{Ba}^{2+}$  currents ( $I_{\text{Ba}}$ ) through recombinant  $\text{Ca}_V$  channels ( $\text{Ca}_V2.2/\beta_3$ ) in HEK-293 cells transiently expressing the wild-type  $\alpha_2\delta$  and its mutants. As can be seen in Fig. 1C, the amplitude of the  $\text{Ba}^{2+}$  currents ( $I_{\text{Ba}}$ ) through  $\text{Ca}_V2.2/\beta_3$  channels, was enhanced by co-expression of the wild-type  $\alpha_2\delta$ -1 subunit, as previously reported (Sandoval *et al.*, 2004; Yasuda *et al.*, 2004). Interestingly, recordings performed in cells transfected with the single (E944Q) and the quadruple (P4) mutations, indicated that the stimulatory effect of the auxiliary subunit was preserved. In contrast, replacement of all six residues in the proteolytic site of the protein significantly decreased whole-cell patch clamp currents (Fig. 1C).

To facilitate comparisons from cells of different size, peak  $I_{\text{Ba}}$  amplitude was normalized to membrane capacitance ( $C_m$ ) and expressed as  $I_{\text{Ba}}$  density (in picoamperes per picofarad). The effects of the wild-type  $\alpha_2\delta$  and its mutant variants E944Q, P4 and P6 on peak  $I_{\text{Ba}}$  density (determined for a test pulse to +10 mV) are summarized in Figure 2A. When compared with the wild-type, E944Q or P4 expressing cells,  $I_{\text{Ba}}$  density in HEK-293 cells transfected with the P6 mutant construct was significantly reduced (>52%).

Figure 2B compares mean  $\pm$  SEM current density-voltage ( $I$ - $V$ ) relationships from HEK-293 cells expressing the wild-type  $\alpha_2\delta$  protein and the E944Q, P4 and P6 mutants. As expected, the E944Q and P4 constructs were not different than wild-type  $\alpha_2\delta$ -1 for effects on  $I_{\text{Ba}}$  density. In contrast, mean peak  $I_{\text{Ba}}$  density in cells expressing the  $\alpha_2\delta$ -1 P6 mutant was severely depressed

across the entire voltage range investigated. In line with this, the macroscopic conductance ( $G_{\max}$ ) for the P6  $\alpha_2\delta$ -1 mutant subunit, determined from the  $I$ - $V$  relationships, was significantly decreased by  $\sim 70\%$  compared with the  $\text{Ca}_V2.2/\beta_3/\alpha_2\delta$ -1 combination. In contrast, a small but no significant inhibition on  $G_{\max}$  was observed by co-expression of the E944Q or P4 mutant subunits (Fig. 3A; Table 1). The  $G_{\max}$  value for the P6 mutant containing channels was comparable to the  $G_{\max}$  value obtained in cells transfected with  $\text{Ca}_V2.2/\beta_3$  in the absence of the  $\alpha_2\delta$ -1 subunit (Table 1), suggesting a role for the proteolytic cleavage of  $\alpha_2\delta$ -1 in surface expression of HVA  $\text{Ca}^{2+}$  channels.

It is well established, that alterations in the maximal conductance in the absence of kinetic changes of the macroscopic currents may depend on variations in the number of functional channels. Therefore, we next investigated whether the effects of the P6 mutation involved alterations in the kinetic properties of the currents recorded. Normalized currents obtained from cells expressing the wild-type  $\alpha_2\delta$  and its mutants showed that the temporal course of the current traces was similar, suggesting that the activation and inactivation rate of the channels were not altered. The channel activation rate was quantitatively compared by fitting current traces to an exponential equation (Sandoval *et al.*, 2004). Accordingly, neither the time to peak nor the time constant for the activation of the current ( $\tau_{\text{act}}$ ) were significantly modified (Fig. 3B). Likewise, we generated complete voltage-dependent inactivation curves for the recombinant  $\text{Ca}_V$  channels expressed. The parameters describing the inactivation curves were similar to the corresponding values obtained from cells expressing the wild-type  $\alpha_2\delta$ -1 (Fig. 4A; Table 1). Lastly, the time constant for the inactivation of the current ( $\tau_{\text{inact}}$ ), obtained by fitting the decaying phase of the currents with an exponential function, and the percentage of current remaining after 140 ms activating pulses were practically indistinguishable between cells expressing the wild-type  $\alpha_2\delta$

and cells expressing the mutant subunits (Fig. 4B; Table 1). Although a decrease in channel open probability cannot be ruled out, our data suggest that the proteolytic cleavage site may be required for cell surface expression.

An interesting finding was that the average current amplitude in the presence of the P6 mutant was comparable to the magnitude of the currents in the absence of the  $\alpha_2\delta$ -1 subunit (Fig. 2). However, the waveforms and the voltage dependence of the currents in the two conditions were different (Figs. 3 and 4). Currents in the absence of any  $\alpha_2\delta$  subunits activate and inactivate significantly slower (Figs. 3B and 4B). Likewise, the half-maximal voltage for current activation and inactivation was shifted  $>5$  mV to the hyperpolarizing direction (Figs. 3A and 4A, *right panels*; Table 1). Taken together, these data suggest that all mutant proteins, including P6 interact with the channel. However, only the P6 mutant is inefficient in trafficking to the cell membrane or is less stable once it reaches the membrane. In keeping with this view, the functional properties of the channels were practically unaltered (Figs. 3 and 4), and only major alterations were observed in the maximal conductance which depends on the number of functional channels in the plasma membrane. Thus conceivably, alterations in the number of  $\text{Ca}_v$  channels at the membrane might be accounting for the effects of the P6  $\alpha_2\delta$ -1 mutant, which might be particularly prone to structural alterations.

## Discussion

In the present report we found that the proteolytic site of the rat brain  $\text{Ca}_v\alpha_2\delta-1$  subunit is localized between amino acid residues Arg-941 and Val-946. Likewise, we found also that a single amino acid substitution in the putative proteolytic site is not sufficient to prevent cleavage, and that replacement of the six residues (Arg-Leu-Leu-Glu-Ala-Val) is required to make the  $\text{Ca}_v\alpha_2\delta-1$  protein insensitive to proteolysis. Furthermore, mutation of the entire hexapeptide prevents the whole-cell current stimulation (normally observed after co-expression of the  $\alpha_2\delta-1$ ) without affecting its kinetic properties, suggesting either changes in channel open probability or a regulation on the number of functional channels at the plasma membrane.

One observation of particular interest in our studies is that hampering normal proteolysis has no biophysical consequences on the  $\text{Ca}_v$  channels regulation by  $\alpha_2\delta-1$ b. Interestingly, recent studies have shown that though an important fraction of the  $\alpha_2\delta-2$  subunit expressed heterologously may not be proteolytically processed into  $\alpha_2-2$  and  $\delta-2$ , it might show full functionality and being expressed on the cell membrane (Douglas *et al.*, 2006). Consequently, it has been suggested that cleavage into  $\alpha_2-2$  and  $\delta-2$  subunits may not be essential for functional enhancement of macroscopic  $\text{Ca}^{2+}$  currents. However, it has been also found that native  $\alpha_2-2$  is fully cleaved in the mouse cerebellum (Douglas *et al.*, 2006), and the possibility exists that the fraction of  $\alpha_2\delta-2$  that is proteolytically cleaved into  $\alpha_2-2$  and  $\delta-2$  in the heterologous system is the active form of the protein. Therefore, it remains an open question for future research whether the  $\alpha_2\delta$  auxiliary subunits are functional in their proteolytically cleaved and non-cleaved forms.

Likewise, previous studies have shown that the  $\text{Ca}_v\alpha_2\delta$  subunits increase the whole-cell conductance in different expression systems (Felix, 1999; Arikath and Campbell, 2003; Klugbauer *et al.*, 2003). Relevant to this, it has been shown that  $\alpha_2\delta-2$  have only a small

influence on the properties of the channels at the single level (Barclay *et al.*, 2001; Brodbeck *et al.*, 2002). Taken together, these data imply that the  $\alpha_2\delta$  subunit is possibly acting on the lifetime of the channel complex at the cell surface, either by enhancing trafficking to the plasma membrane or by reducing turnover of channels (Douglas *et al.*, 2006). In agreement with this view, it has been reported that the  $\alpha_2\delta$  subunits increase the amount of  $\text{Ca}_v1$  and  $\text{Ca}_v2$  proteins in the cell membrane (Felix, 1999; Arikath and Campbell, 2003; Klugbauer *et al.*, 2003).

Although the precise mechanism by which the  $\alpha_2\delta$  subunit controls  $\text{Ca}^{2+}$  channel turnover is presently unclear, Bernstein and Jones (2006) have recently suggested a model where  $\alpha_2\delta$  serves to maintain  $\text{Ca}_v2.2$  channel levels at the cell surface by limiting their entry into a degradative pathway. According to this model, any free (possibly less physiologically desirable)  $\text{Ca}_v2.2/\beta$  complexes at the cell surface, are more rapidly removed and diverted to a non-degradable pool, perhaps for further attempts at complexation with  $\alpha_2\delta$  subunits (Bernstein and Jones, 2006). Therefore, investigating to what extent proteolytic processing alterations of  $\alpha_2\delta$  interfere with  $\text{Ca}_v$  channel subunit proper folding and trafficking is an interesting topic for future studies.

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## References

1. Arikath J, Campbell KP. Auxiliary subunits: essential components of the voltage-gated calcium channel complex. *Curr Opin Neurobiol* 2003;13:298-307.
2. Avila G, Sandoval A, Felix R. (2004) Intramembrane charge movement associated with endogenous K<sup>+</sup> channel activity in HEK-293 cells. *Cell Mol Neurobiol* 24, 317-330.
3. Bangalore R, Mehrke G, Gingrich K, Hofmann F, Kass RS. (1996) Influence of L-type Ca channel  $\alpha_2\delta$  subunit on ionic and gating current in transiently transfected HEK 293 cells. *Am J Physiol* 270, H1521-H528.
4. Barclay J, Balaguero N, Mione M, Ackerman SL, Letts VA, Brodbeck J, Canti C, Meir A, Page KM, Kusumi K, PerezReyes E, Lander ES, Frankel WN, Gardiner RM, Dolphin AC, Rees M. (2001) Ducky mouse phenotype of epilepsy and ataxia is associated with mutations in the *Cacna2d2* gene and decreased calcium channel current in cerebellar Purkinje cells. *J Neurosci* 21, 6095-6104.
5. Bernstein GM, Jones OT. (2006) Kinetics of internalization and degradation of N-type voltage-gated calcium channels: Role of the  $\alpha_2/\delta$  subunit. *Cell Calcium* (in press).
6. Brodbeck J, Davies A, Courtney J-M, Meir A, Balaguero N, Canti C, Moss FJ, Page KM, Pratt WS, Hunt SP, Barclay J, Rees M, Dolphin AC (2002) The ducky mutation in *Cacna2d2* results in altered Purkinje cell morphology and is associated with the expression of a truncated  $\alpha_2\delta$ -2 protein with abnormal function. *J Biol Chem* 277, 7684-7693.
7. Canti C, Nieto-Rostro M, Foucault I, Heblich F, Wratten J, Richards MW, Hendrich J, Douglas L, Page KM, Davies A, Dolphin AC. The metal-ion-dependent adhesion site in

- the Von Willebrand factor-A domain of  $\alpha_2\delta$  subunits is key to trafficking voltage-gated  $\text{Ca}^{2+}$  channels. *Proc Natl Acad Sci USA*. 2005; 102, 11230-11235.
8. Castellano A, Wei X, Birnbaumer L, Perez-Reyes E. Cloning and expression of a neuronal calcium channel  $\beta$  subunit. *J. Biol. Chem.* 1993; 268, 3450-3455.
  9. Catterall WA, Perez-Reyes E, Snutch TP, Striessnig J. International Union of Pharmacology. XLVIII. Nomenclature and structure-function relationships of voltage-gated calcium channels. *Pharmacol Rev* 2005; 57:411-425.
  10. De Jongh KS, Warner C, Catterall WA. (1990) Subunits of purified calcium channels.  $\alpha_2$  and  $\delta$  are encoded by the same gene. *J Biol Chem* 265, 14738-14741.
  11. Douglas L, Davies A, Wratten J, Dolphin AC. (2006) Do voltage-gated calcium channel  $\alpha_2\delta$  subunits require proteolytic processing into  $\alpha_2$  and  $\delta$  to be functional? *Biochem Soc Trans* 34(Pt 5), 894-898.
  12. Ellis SB, Williams ME, Ways NR, Brenner R, Sharp AH, Leung AT, Campbell KP, McKenna E, Koch WJ, Hui A, Schwartz A, Harpold MM. (1988) Sequence and expression of mRNAs encoding the  $\alpha_1$  and  $\alpha_2$  subunits of a DHP-sensitive calcium channel. *Science* 241, 1661-1664.
  13. Felix R, Gurnett CA, De Waard M, Campbell KP. (1997) Dissection of functional domains of the voltage-dependent  $\text{Ca}^{2+}$  channel  $\alpha_2\delta$  subunit. *J Neurosci* 17, 6884-6891.
  14. Felix R. (1999) Voltage-dependent  $\text{Ca}^{2+}$  channel  $\alpha_2\delta$  auxiliary subunit: structure, function and regulation. *Receptors Channels* 6, 351-362.
  15. Felix R. (2005) Molecular regulation of voltage-gated  $\text{Ca}^{2+}$  channels. *J Recept Signal Transduct Res* 25:57-71.



16. Fujita Y., Mynlieff M., Dirksen R.T., Kim M.S., Niidome T., Nakai J., Friedrich T., Iwabe N., Miyata T., Furuichi T., Furutama D., Mikoshiba K., Mori Y., Beam K.G. Primary structure and functional expression of the  $\omega$ -conotoxin-sensitive N-type calcium channel from rabbit brain. *Neuron*. 10 (1993) 585-598.
17. Gurnett CA, De Waard M, Campbell KP. (1996) Dual function of the voltage-dependent  $\text{Ca}^{2+}$  channel  $\alpha_2\delta$  subunit in current stimulation and subunit interaction. *Neuron* 16, 431-440.
18. Gurnett CA, Felix R, Campbell KP. (1997) Extracellular interaction of the voltage-dependent  $\text{Ca}^{2+}$  channel  $\alpha_2\delta$  and  $\alpha_1$  subunits. *J Biol Chem* 272, 18508-18512.
19. Hamill OP, Marty A, Neher E, Sakmann B, Sigworth FJ (1981) Improved patch-clamp techniques for high resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* 391, 85-100.
20. Hobom M, Dai S, Marais E, Lacinova L, Hofmann F, Klugbauer N. (2000) Neuronal distribution and functional characterization of the calcium channel  $\alpha_2\delta$ -2 subunit. *Eur J Neurosci* 12, 1217-1226.
21. Jay SD, Sharp AH, Kahl SD, Vedvick TS, Harpold MM, Campbell KP. (1991) Structural characterization of the dihydropyridine-sensitive calcium channel  $\alpha_2$ -subunit and the associated  $\delta$  peptides. *J Biol Chem* 266, 3287-3293.
22. Jones LP, Wei SK, Yue DT. (1998) Mechanism of auxiliary subunit modulation of neuronal  $\alpha_{1E}$  calcium channels. *J Gen Physiol* 112, 125-143.
23. Kim H.L., Kim H., Lee P., King R.G., Chin H. Rat brain expresses an alternatively spliced form of the dihydropyridine-sensitive L-type calcium channel  $\alpha_2$  subunit. *Proc Natl Acad Sci* 89 (1992) 3251-3255.

24. Klugbauer N, Lacinova L, Marais E, Hobom M, Hofmann F. (1999) Molecular diversity of the calcium channel  $\alpha_2\delta$  subunit. *J Neurosci* 19, 684-691.
25. Klugbauer N, Marais E, Hofmann F. (2003) Calcium channel  $\alpha_2\delta$  subunits: differential expression, function, and drug binding. *J Bioenerg Biomembr* 35, 639-647.
26. Lacinova L. Voltage-dependent calcium channels. *Gen Physiol Biophys* 2005;24 Suppl 1:1-78.
27. Obermair GJ, Kugler G, Baumgartner S, Tuluc P, Grabner M, Flucher BE. The  $\text{Ca}^{2+}$  channel  $\alpha_2\delta$ -1 subunit determines  $\text{Ca}^{2+}$  current kinetics in skeletal muscle but not targeting of  $\alpha_{1S}$  or excitation-contraction coupling. *J Biol Chem*. 2005;280, 2229-2237.
28. Qin N, Olcese R, Stefani E, Birnbaumer L. (1998) Modulation of human neuronal  $\alpha_1E$ -type calcium channel by  $\alpha_2\delta$ -subunit. *Am J Physiol* 274, C1324-C1331.
29. Qin N, Yagel S, Momplaisir ML, Codd EE, D'Andrea MR. (2002) Molecular cloning and characterization of the human voltage-gated calcium channel  $\alpha_2\delta$ -4 subunit. *Mol Pharmacol* 62, 485-496.
30. Sandoval A, Oviedo N, Andrade A, Felix R. (2004) Glycosylation of asparagines 136 and 184 is necessary for the  $\alpha_2\delta$  subunit-mediated regulation of voltage-gated  $\text{Ca}^{2+}$  channels. *FEBS Lett* 576, 21-26.
31. Shirokov R, Ferreira G, Yi J, Rios E. (1998) Inactivation of gating currents of L-type calcium channels. Specific role of the  $\alpha_2\delta$  subunit. *J Gen Physiol* 111, 807-823.
32. Shistik E, Ivanina T, Pury T, Hosey M, Dascal N. (1995)  $\text{Ca}^{2+}$  current enhancement by  $\alpha_2\delta$  and  $\beta$  subunits in *Xenopus* oocytes: contributions of changes in channel gating and  $\alpha_1$  protein level. *J. Physiol.* 489:55-62.

33. Singer D, Biel M, Lotan I, Flockerzi V, Hofmann F, Dascal N. (1991) The roles of the subunits in the function of the calcium channel. *Science* 253, 1553-1557.
34. Welling A, Bosse E, Cavalie A, Bottlender R, Ludwig A, Nastainczyk W, Flockerzi V, Hofmann F. (1993) Stable co-expression of calcium channel  $\alpha_1$ ,  $\beta$  and  $\alpha_2/\delta$  subunits in a somatic cell line. *J Physiol* 471, 749-765.
35. Yasuda T, Chen L, Barr W, McRory JE, Lewis RJ, Adams DJ, Zamponi GW. (2004) Auxiliary subunit regulation of high-voltage activated calcium channels expressed in mammalian cells. *Eur J Neurosci*. 20, 1-13.

**FIGURE 1**

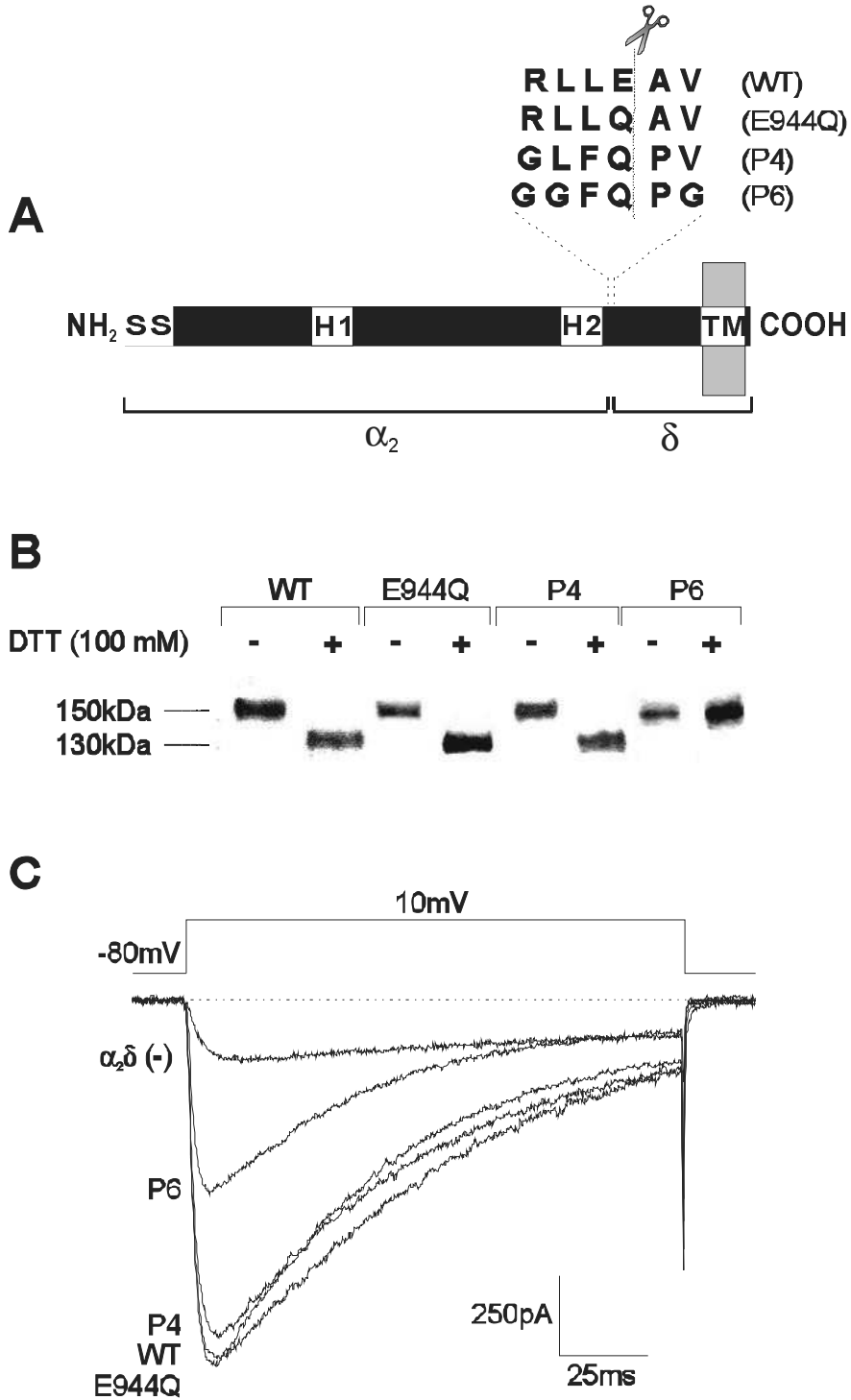


FIGURE 2

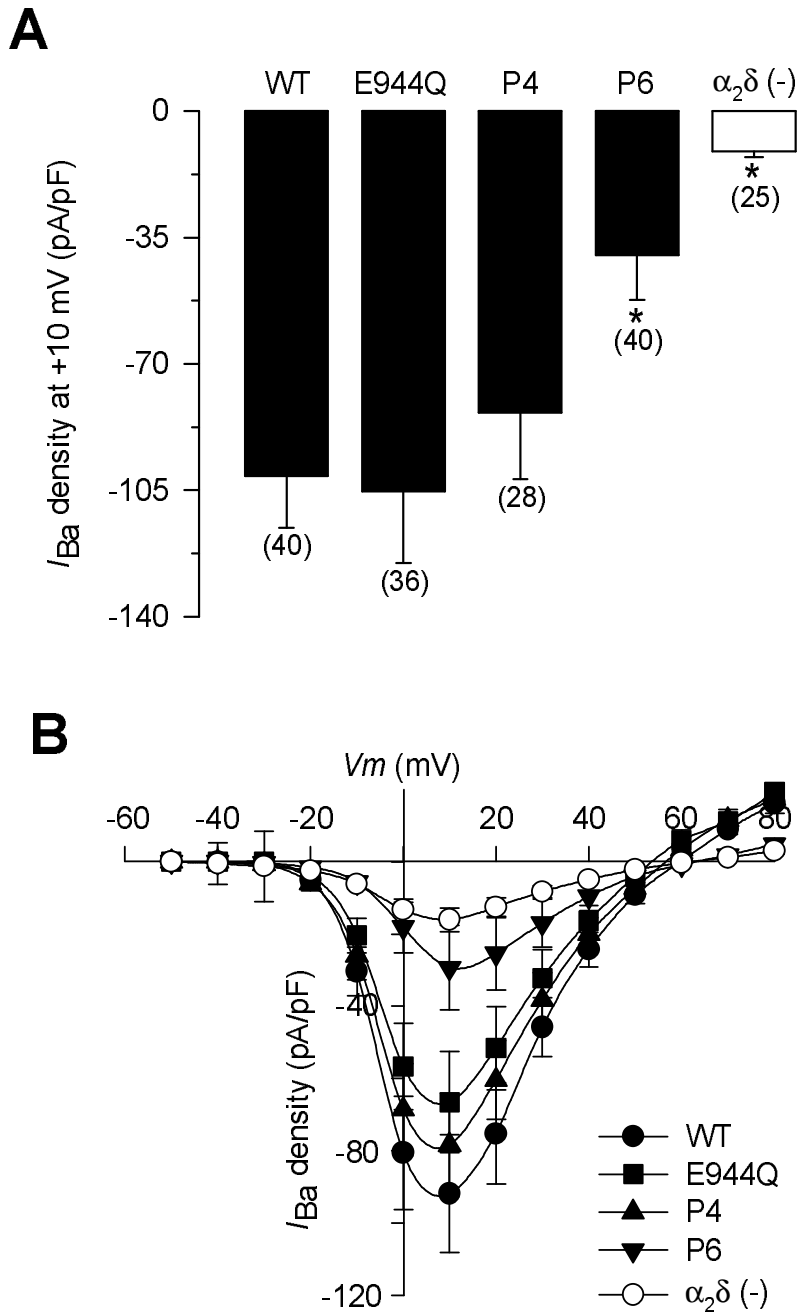


FIGURE 3

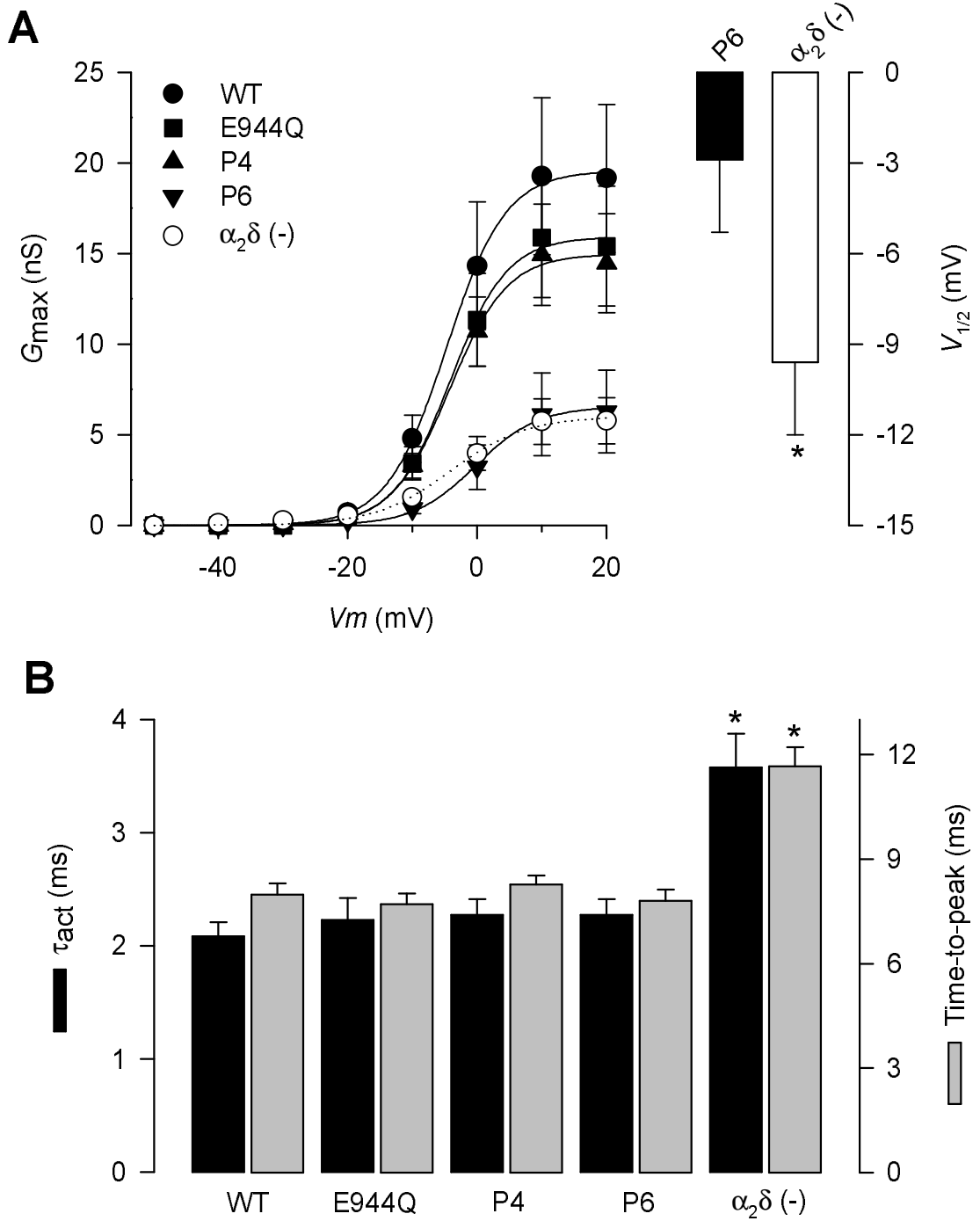


FIGURE 4

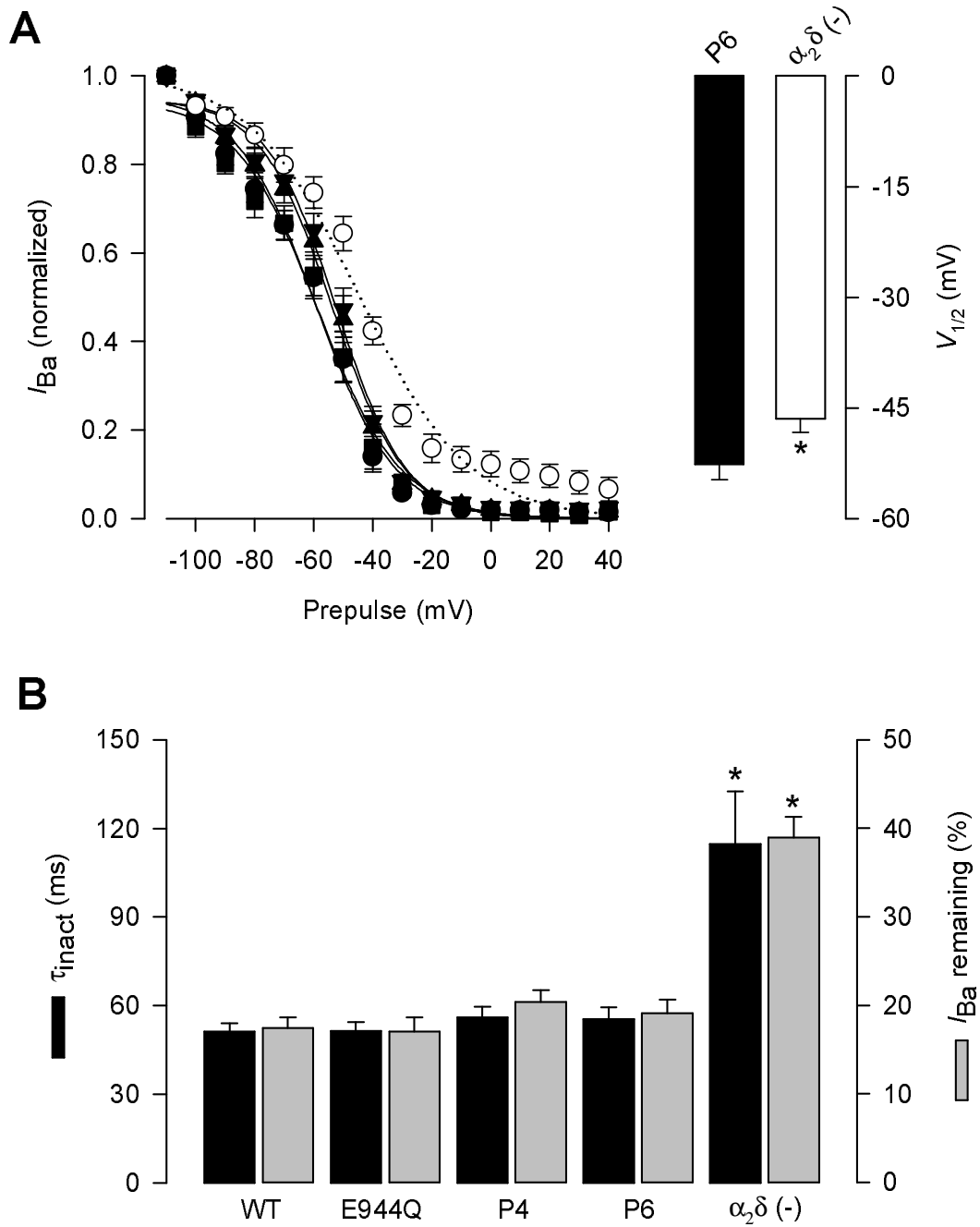


Table 1. Differential effects of the wild-type  $\alpha_2\delta$  subunit and its proteolysis mutants on the biophysical properties of the  $\text{Ca}_v2.2/\beta_3$  currents

	Wild-type	E944Q	P4	P6	$\alpha_2\delta$ -1 (-)
Potential for half-activation (mV)	-4.5.3±1.8	-4.0±0.9	-4.7±1.8	-3.0±2.4	-9.6±2.4
Maximal conductance at +10 mV (nS)	19.3±4.3	15.9±3.3	14.9±2.8	6.1±2.3	5.7±1.3
Potential for half steady-state inactivation (mV)	-60.2±3.6	-59.7±4.9	-56.8±3.0	-52.3±2.2	-44.6±2.1
Rate of inactivation at +10 mV ( $\mu\text{s}^{-1}$ )	20.4±1.2	20.1±1.0	18.9±1.1	19.6±1.4	12.3±1.1



**Figure legends**

**Figure 1.** Expression of the  $\alpha_2\delta$  subunit proteolytic site mutants in HEK-293 cells. A) Schematic representation of the  $\text{Ca}_v\alpha_2\delta$  auxiliary subunit. The location of the two hydrophobic domains (H1 and H2), and the signal sequence (S) are given. The amino acid residues in the putative proteolytic site of the wild-type  $\alpha_2\delta$ -1 and its mutant versions are aligned and shown above the diagram. B) Western blot analysis of membranes from HEK-293 cells expressing the wild-type, and the mutant versions of the  $\alpha_2\delta$  subunit (E944Q, P4 and P6). Membranes were prepared as described under Material and Methods. Only the sextuple mutation (P6) is insensitive to proteolysis (do not change its electrophoretic pattern after dithiothreitol -DDT- treatment). C) Representative  $\text{Ba}^{2+}$  currents through recombinant  $\text{Ca}_v2.2/\beta_3$  channels obtained in HEK-293 cells co-expressing the wild-type  $\alpha_2\delta$ -1 and its proteolysis mutants. Currents were elicited in response to 140 ms test pulses to +10 mV delivered from a holding potential of -80 mV.

**Figure 2.** Functional effects of heterologous expression of the wild-type  $\alpha_2\delta$  subunit and its proteolytic site mutants. A) Average  $\pm$  SEM  $I_{\text{Ba}}$  density in HEK-293 cells expressing the wild-type  $\alpha_2\delta$ -1 and its mutant variants (filled bars).  $I_{\text{Ba}}$  density was calculated from  $I_{\text{Ba}}$  amplitude at a test pulse of +10 mV normalized to cell membrane capacitance. 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 ( $P < 0.05$ ). B) Average  $\pm$  SEM  $I$ - $V$  relationships for  $I_{\text{Ba}}$  recorded from HEK-293 cells expressing the wild-type  $\alpha_2\delta$ -1 and its mutant variants (E944Q, P4 and P6).  $I_{\text{Ba}}$  density was calculated at a series of test pulses applied from a holding potential of -80 mV in 10 mV steps between -50 and +80 mV ( $n = 6-8$ ). Open circles represent the average current density in the absence of the  $\alpha_2\delta$  subunit.

**Figure 3.** *Activation properties of  $Ca_v2.2/\beta_3$  channels co-expressed with the wild-type  $\alpha_2\delta-1$  or its proteolysis mutations.* A) Left, voltage dependence of activation of  $Ca_v2.2/\beta_3$  channels co-expressed with wild-type  $\alpha_2\delta-1$  (filled circles), its proteolysis mutants (E944Q, P4 and P6) or without any  $\alpha_2\delta$  subunit (open circles). Maximum conductance ( $G_{max}$ ) plotted data were derived from Fig. 2B. The mean data were fitted with Boltzmann functions, the  $V_{1/2}$  values of which are given in Table 1. Right, comparison of the mean half-activation voltage ( $V_{1/2}$ ) for  $Ca_v2.2/\beta_3$  channels co-expressed with the P6 proteolysis mutation or without the  $\alpha_2\delta$  subunit ( $n = 6-8$ ). B) Mean time to peak and time constants of activation ( $\tau_{act}$ ) obtained by fitting the rising phase of  $I_{Ba}$  at +10 mV with a single exponential, for  $Ca_v2.2/\beta_3$  channels co-expressed with wild-type  $\alpha_2\delta-1$ , its proteolysis mutants, or without any  $\alpha_2\delta$  subunit ( $n = 11-25$ ).

**Figure 4.** *Inactivation properties of  $Ca_v2.2/\beta_3$  channels co-expressed with the wild-type  $\alpha_2\delta-1$  or its proteolysis mutations.* A) Left, voltage dependence of steady-state inactivation of  $Ca_v2.2/\beta_3$  channels co-expressed with wild-type  $Ca_v\alpha_2\delta-1$  (filled circles), its proteolysis mutants E944Q, P4 and P6 or without any  $\alpha_2\delta$  subunit (open circles). Currents were recorded after conditioning pulses of 1 s duration, applied from a holding potential of -80 mV in 10 mV steps between -110 and +40 mV, followed by a 140 ms test pulse to +10 mV. The normalized data are plotted against the conditioning potentials ( $n = 5-11$ ). The mean data were fitted with a Boltzmann function, the  $V_{1/2}$  values of which are given in Table 1. *Right*, comparison of the mean half-inactivation voltage ( $V_{1/2}$ ) for  $Ca_v2.2/\beta_3$  channels co-expressed with the P6 mutation or without the  $\alpha_2\delta$  subunit. B) Average time constants of inactivation ( $\tau_{inact}$ ) and percentage of current remaining 140 ms into the depolarizing pulse for  $Ca_v2.2/\beta_3$  channels co-expressed with wild-type  $\alpha_2\delta-1$ , its proteolysis mutants, or without any  $\alpha_2\delta$  subunit ( $n = 11-25$ ).