

Two PEST-like motifs regulate Ca^{2+} /calpain-mediated cleavage of the $\text{Ca}_v\beta_3$ subunit and provide important determinants for neuronal Ca^{2+} channel activity

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Abstract

Increase in intracellular Ca^{2+} due to voltage-gated Ca^{2+} (Ca_v) channel opening represents an important trigger for a number of second-messenger mediated effects ranging from neurotransmitter release to gene activation. Ca^{2+} entry occurs through the principal pore-forming protein, but several ancillary subunits are known to more precisely tune ion influx. Among them, the $\text{Ca}_v\beta$ subunits are perhaps the most important given that they largely influence the biophysical and pharmacological properties of the channel. Notably, several functional features may be associated with specific structural regions of the $\text{Ca}_v\beta$ subunits emphasizing the relevance of intramolecular domains in the physiology of these proteins. In the current report, we show that $\text{Ca}_v\beta_3$ contains two PEST motifs and undergoes Ca^{2+} -dependent degradation which can be prevented by the specific calpain inhibitor calpeptin. Using mutant constructs lacking the PEST motifs, we present evidence that they are necessary for the cleavage of $\text{Ca}_v\beta_3$ by calpain. Furthermore, the deletion of the PEST sequences did not affect the binding of $\text{Ca}_v\beta_3$ to the ion-conducting $\text{Ca}_v2.2$ subunit, and when expressed in HEK-293 cells, the PEST motif-deleted $\text{Ca}_v\beta_3$ significantly increased whole-cell current density and retarded channel inactivation. Consistent with this observation, calpeptin treatment of HEK-293 cells expressing wild-type $\text{Ca}_v\beta_3$ resulted in an increase in current amplitude. Together, these findings suggest that calpain-mediated $\text{Ca}_v\beta_3$ proteolysis may be an essential process for Ca^{2+} channel functional regulation.

Introduction

The major function of the voltage-gated Ca^{2+} (Ca_V) channels is to convert changes in membrane potential into an intracellular calcium (Ca^{2+}_i) signal. Transient rises of Ca^{2+}_i trigger or regulate diverse intracellular events, including metabolic processes, muscle contraction, secretion of hormones and neurotransmitters, cell differentiation and gene expression. Several types of Ca_V channels have been characterized and designated L, N, P/Q, R, and T. These channel types can be grouped into two major functional classes: high voltage- and low voltage-activated channels (HVA and LVA, respectively). The HVA Ca_V channel permeation pathway is formed by its α_1 subunit, which is encoded by a family of 7 genes (Catterall *et al.*, 2003). The current through these channels may be modulated by distinct structural modifications including the association with auxiliary subunits: the disulfide-linked $\text{Ca}_V\alpha_2\delta$, the intracellular $\text{Ca}_V\beta$ and the transmembrane $\text{Ca}_V\gamma$ subunits, which also represent gene families (Arikkath and Campbell, 2003).

Among the auxiliary proteins, the $\text{Ca}_V\beta$ subunit plays a crucial role in the formation and behavior of all functional HVA Ca_V channels. Four different types of $\text{Ca}_V\beta$ subunits (β_1 to β_4) have been identified, each with multiple splicing variants (Walker and De Waard, 1998; Arikkath and Campbell, 2003; Dolphin, 2003). The $\text{Ca}_V\beta$ proteins do not cross the plasma membrane, but can directly interact with the $\text{Ca}_V\alpha_1$ subunit and are important for trafficking and expression of the kinetic properties of the channel (Walker and De Waard, 1998; Arikkath and Campbell, 2003; Dolphin, 2003). The physiological importance of the $\text{Ca}_V\beta$ subunits is demonstrated by the severe phenotypes of mutant and knockout mice (Gregg *et al.*, 1996; Burgess *et al.*, 1997; McEnery *et al.*, 1999; Ball *et al.*, 2002). In-depth understanding of how the $\text{Ca}_V\beta$ auxiliary subunits modulate

the activity of the ion-conducting $\text{Ca}_v\alpha_1$ subunits is essential for insights into the operation of HVA Ca_v channels in both normal and disease states.

Detailed structural modeling of $\text{Ca}_v\beta$ subunits has proposed five discrete domains homologous to the membrane-associated guanylate kinase (MAGUK) protein family. Structural models have been proposed for the type 3 src-homology (SH3), and guanylate kinase (GK)-like domains (Hanlon *et al.*, 1999; Opatowsky *et al.*, 2003; 2004; McGee *et al.*, 2004; Takahashi *et al.*, 2004; Chen *et al.*, 2004;). SH3 and GK domains are both necessary to recapitulate full modulatory effects of $\text{Ca}_v\beta$ on the pore-forming $\text{Ca}_v\alpha_1$ protein (Takahashi *et al.*, 2004; McGee *et al.*, 2004). Likewise, the C-terminus of $\text{Ca}_v\beta$ is associated to membrane targeting properties (Bogdanov *et al.*, 2000) and the N-terminus appears to be involved in the regulation of channel inactivation (Olcese *et al.*, 1994; Restituito *et al.*, 2000; Stotz *et al.*, 2004). Site-directed mutagenesis of conserved serine residues in consensus sites for protein kinase phosphorylation suggests a role of phosphorylation in tuning the functional properties and pharmacological sensitivities of the $\text{Ca}_v\beta$ subunits (De Waard *et al.*, 1994; Gerhardstein *et al.*, 1999; Kohn *et al.*, 2003).

These studies point to the importance of intramolecular domains in the physiology of the $\text{Ca}_v\beta$ subunits. In the current report, we show that $\text{Ca}_v\beta_3$ contains two PEST-like sequences (potential signals for rapid protein degradation), one in the SH3 domain and another in the C-terminal end of the protein. These sequences are sensitive *in vitro* to low concentrations of calpain (a Ca^{2+} -dependent protease). We further found that $\text{Ca}_v\beta_3$ mutants lacking the PEST sequences induced an increase in whole-cell Ca^{2+} current amplitude compared to wild-type $\text{Ca}_v\beta_3$, and caused a change in channel inactivation kinetic properties. Our findings suggest that $\text{Ca}_v\beta_3$ proteolytic cleavage may be an essential process for Ca^{2+} channel functional regulation.

Materials and Methods

Cell culture and recombinant Ca_v channel expression

Human embryonic kidney (HEK-293) cells were grown in DMEM-high glucose supplemented with 10% horse serum, 2 mM L-glutamine, 110 mg/l sodium pyruvate and 50 μ g/ml gentamycin, at 37°C in a 5% CO₂/95% air humidified atmosphere. After splitting the cells on the previous day and seeding at ~60% confluency, cells were transfected using the Lipofectamine Plus reagent (Gibco BRL) with 1.2 μ g plasmid cDNA encoding the rabbit brain N-type Ca²⁺ channel Cav2.2 pore-forming subunit (formerly α_{1B} ; GenBank accession number D14157) (Fujita *et al.*, 1993) in combination with 1.2 μ g cDNA coding the rat brain Cav $\alpha_2\delta$ -1 (M86621) (Kim *et al.*, 1992), and 1.2 μ g cDNA of the rat brain Cav β_3 (M88751) (Castellano *et al.*, 1993) or its mutants (see below). For electrophysiology, 0.36 μ g of a plasmid cDNA encoding the green fluorescent protein (GFP; Green-Lantern; Gibco/BRL) was added to the transfection mixture to select positively transfected cells.

Deletions of Cav β_3 PEST regions

The different deletions in the Cav β_3 subunit were obtained by the QuikChange XL-mutagenesis kit QCM (Stratagene), following a two stage PCR protocol for deletions (Wang and Malcolm, 2001). The PEST regions 1 and 2 (amino acid residues 24 to 37 and 397 to 411, respectively) were subjected to deletion through duplex oligonucleotides that comprised the adjacent nucleotidic sequences to these regions. The forward oligonucleotides were 5'-GTTCAGCCGACTCTACACCAGAGAGTGCCCGGCGAGAAGTGG-3' and 5'-GAGGAGCATTACCCCCTGGAGCAGGCCTGGACCGGATCTTCACAG-3' for PEST1 and PEST2, respectively. Reverse oligonucleotides were complementary to these sequences. In step I of the procedure, two extension reactions were performed in separate tubes, one containing the

forward primer and the other including the reverse and complementary primer. Five polymerization cycles were conducted at 95°C 30 s, 55°C 1 min, and 68°C 14 min. After that, both reactions were mixed and the standard QCM procedure continued for 16 cycles. In addition to the single PEST deletions, a double deletion cDNA clone was obtained from the first plasmid harboring the PEST1 deletion in combination with the duplex oligonucleotides for the PEST2 deletion. Deletions were confirmed by either restriction endonuclease analysis (using endonucleases *HindIII* and *XbaI*) or automatic sequencing using an ABI PRISM 310 sequence analyzer (Perkin-Elmer Applied Biosystems) and primers for the T7 and SP6 promoters.

In vitro transcription and translation

In vitro transcription/translation assays were performed using the TNT™ Quick Coupled transcription/translation system kit (Promega). Briefly, 2 µg of plasmid DNA was added to 41 µl of TNT™ quick master Mix containing 1 µl of [³⁵S]-methionine (1000 Ci/mmol) at 2.5 mCi/ml (Amersham Pharmacia Biotech) to a final volume of 50 µl and incubated at 30°C for 120 min. Proteins were subjected to SDS-PAGE (see below) and labeled proteins were detected by film exposure for 48 h.

SDS-PAGE and Western blotting

Microsomes from transfected HEK-293 cells were obtained as described elsewhere (Felix *et al.*, 1997; Gurnett *et al.*, 1997), and proteins were separated on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels according to the method of Laemmli (1970). Samples were heated at 90°C for 5 min and 100 µg of protein/slot were loaded on gels. Proteins were blotted onto nitrocellulose membranes and were developed with enhanced chemiluminescence as previously described (Felix *et al.*, 1997; Gurnett *et al.*, 1997). The anti-Cavβ₃ specific antibody was a sheep

polyclonal antibody (1:1000 dilution; Sh0049), and the secondary antibody was a rabbit anti-sheep IgG horseradish peroxidase (Zymed) used at a dilution of 1:4000.

Pull-down experiments

As mentioned earlier, [³⁵S]-labelled proteins (wild-type β_3 , $\beta_3\Delta P1$, $\beta_3\Delta P2$ and $\beta_3\Delta P1-2$) were expressed *in vitro* using a coupled transcription/translation system as indicated by the manufacturer (TNTTM Kit, Promega). For pull-down assays, 75 μ l of hydrated glutathione-agarose beads (Sigma) were incubated respectively with 20 μ g of GST fused to AID_{2.2} (Alpha1 Interaction Domain of Ca_v2.2; Sandoz *et al.*, 2001) or of GST alone for 2 hours at 4°C. In order to saturate non specific free sites, GST-AID_{2.2} and GST beads were incubated with 0.1 mg/ml BSA overnight at 4°C. [³⁵S]-labelled proteins were incubated with the beads for 1 h at room temperature. The beads were then washed with PBS three times and the proteins, bound to beads, were eluted in denaturing buffer and analyzed on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by autoradiography.

Electrophysiology

Forty eight hours after transfection, cells expressing the GFP reporter gene were subjected to the whole-cell mode of the patch clamp technique (Hamill *et al.*, 1981). In brief, Ba²⁺ currents through Ca²⁺ channels 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 and by 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. The bath recording solution contained (in mM) 10 BaCl₂, 125 TEA-Cl, 10 HEPES and 10 glucose (pH 7.3). The internal solution consisted of (in mM) 110 CsCl, 5 MgCl₂, 10 EGTA, 10 HEPES, 4 Na-ATP and 0.1 GTP (pH 7.3). Experiments were performed at room temperature (~25°C).

Pulse chase and immunoprecipitation experiments

6 cm diameter dishes with 40% confluent HEK-293 cells were transfected with cDNA encoding the wild-type Cavβ₃ or its ΔPEST mutants. 24 hours later, the protein labeling condition was set by incubating the cells for 30 min in methionine- and cysteine-free DMEM supplemented with 5% fetal calf serum (FCS; starvation period). Labeling was induced by adding 500 μCi [³⁵S]-L-methionine and 2 mM L-cysteine to each plate for 40 min at 37°C. To remove radioactive media, dishes were washed with PBS. Subsequently, normal DMEM media supplemented with 10% FCS was added to plates (except the t = 0 sample, which represents the start time of the chase). At t = 24 h, all plates were washed with ice-cold PBS. Cells were then scraped in 10 ml ice-cold PBS and transferred to a tube to remove the supernatant by centrifugation. Labeled cells were lysed with 1 ml PBS supplemented with 0.5% triton X-100 and a cocktail of protease inhibitors (complete, Mini, EDTA-free, Roche). Lysates were sonicated and centrifuged at 1,500 rpm for 15 min. The supernatants were used for immunoprecipitation experiments. A total of 200 μg proteins of each sample were incubated with anti-Cavβ₃ polyclonal IgG for 1 hr at room temperature. This polyclonal antibody was described elsewhere (Bichet *et al.*, 2000), but was raised against the full-length sequence of Cavβ₃. Subsequently, the Cavβ₃-IgG complex were immobilized by Protein A sepharose beads. Eluted proteins were then loaded on a 12% SDS-PAGE. After protein separation, the gel was treated for 30 min with a fixation solution (50%

methanol, 10% acetic acid) and 30 min with a solution of 10% glycerol, before a 24 hr autoradiography exposure.

Data analysis

The data are given as mean \pm S.E. Statistical differences between two means were determined by Student's t tests ($P < 0.05$). Current inactivation was fitted with single exponential equations of the form: $A \times \exp(-t/\tau) + c$, where A is the initial amplitude (pA), t is time (ms), τ is the time constant for inactivation and c is a constant.

Results

Although important progresses in the structure-function relationship of the $\text{Ca}_v\beta$ subunits have been made recently (Richards *et al.*, 2004), almost nothing is known about the mechanism and determinants of recognition for proteolytic degradation of these proteins. In this context, PEST domains are short sequences (10-60 residues) enriched in proline (P), glutamic/aspartic (E), serine (S) and threonine (T) residues, found in many short-lived eukaryotic proteins that play a role in their degradation (Rechsteiner and Rogers, 1996). Using the PESTFind algorithm (available at the URL <https://embl.bcc.univie.ac.at/toolbox/>) we found one conserved PEST sequence (N-terminal; PEST1) and one variable (C-terminal; PEST2) PEST sequence in the $\text{Ca}_v\beta$ proteins. In the rat brain $\text{Ca}_v\beta_3$ subunit, the PEST1 region comprises amino acid residues 24 to 37, while PEST2 consists of amino acid residues 397 to 411 (Fig. 1A). PEST1 scored +9.47 and PEST2 +11.52, respectively. These findings prompted us to investigate the importance of the PEST sequences in the susceptibility of $\text{Ca}_v\beta_3$ to proteolytic degradation. It is worth mentioning that the $\text{Ca}_v\beta_3$ subunit is the major $\text{Ca}_v\beta$ constituent of the $\text{Ca}_v2.2$ channel complex (Scott *et al.*, 1996), which plays a pivotal role in regulating neurotransmitter release and in controlling endocrine secretion.

By using a two stages PCR protocol (see Methods section), we first created two single (ΔP1 and ΔP2) and one double ($\Delta\text{P1-2}$) PEST domain deletions in the $\text{Ca}_v\beta_3$ sequence. The plasmids containing the full-length sequence and the constructs harboring the deletions were initially examined using a cell-free transcription/translation system. All the cDNA clones directed the synthesis *in vitro* of polypeptides of the expected molecular weights (Fig. 1B). Next, the wild-type $\text{Ca}_v\beta_3$ and its three PEST-deleted versions were expressed in HEK-293 cells and analyzed by immunoblotting using polyclonal antibodies directed against a fusion protein of the C-

terminus of $\text{Ca}_v\beta_3$ (Liu *et al.*, 1996; Scott *et al.*, 1996). This analysis revealed a single immunoreactive protein band with a molecular mass of ~58 kDa in microsomes from transfected cells, which corresponds to the full-length $\text{Ca}_v\beta_3$ (Fig. 1C). The antibodies also recognized the PEST-truncated proteins by showing shifts in the mobility from 58 kDa down to ~50-55 kDa on SDS-PAGE 10% gels (Fig. 1C). These findings, combined with the lack of endogenous $\text{Ca}_v\beta_3$ subunit, make the HEK-293 cell line a good cell model to investigate the importance of the PEST sequences in $\text{Ca}_v\beta_3$ function.

We next questioned whether the PEST domains of $\text{Ca}_v\beta_3$ were molecular substrates for *in vitro* degradation by Ca^{2+} -dependent endogenous proteases. This was tested through a comparative analysis of Ca^{2+} -mediated proteolysis using the $\text{Ca}_v\beta_3$ mutant lacking both PEST regions ($\Delta\text{P1-2}$) and the full-length $\text{Ca}_v\beta_3$ subunit as substrates. Figure 2A shows that recombinant $\text{Ca}_v\beta_3$ expressed in HEK-293 cells was partially cleaved by endogenous proteases in the presence of CaCl_2 . Though significant degradation was observed, proteolysis was not complete; this could be explained by the fact that besides Ca^{2+} no other agent was used to induce proteolysis. In addition, endogenous molecules that activate proteolytic activity (by reducing the Ca^{2+} requirement, for instance), may be lacking in the cell homogenates employed in these assays. Hence, when Ca^{2+} was present, the specific anti- $\text{Ca}_v\beta_3$ antibody detected additional bands that should correspond to $\text{Ca}_v\beta_3$ fragments following the cleavage of the full-length $\text{Ca}_v\beta_3$ by Ca^{2+} -dependent endogenous proteases. As expected, this proteolytic break-down of $\text{Ca}_v\beta_3$ was prevented by adding EDTA to the incubation buffer used for the experiments. Unlike the full-length $\text{Ca}_v\beta_3$ subunit, the $\Delta\text{P1-2}$ mutant was stable in the presence of Ca^{2+} and did not undergo proteolysis in the EDTA-containing buffer (Fig. 2A). Likewise, examination of gels stained with Coomassie Blue provided initial evidence that a ~58,000-Da polypeptide (presumably the $\text{Ca}_v\beta$)

is susceptible to Ca^{2+} /calpain-induced proteolysis. Hence, microsomes of HEK-293 cells expressing the $\text{Ca}_v\beta_3$ subunit were analyzed on 10% SDS-PAGE stained with Coomassie Blue. As shown in Figure 2B, a 20 min treatment of the microsomes with μ -calpain produced a dose-dependent change in the levels of the ~58 kDa protein in the gels. In contrast, the levels of the ~58 kDa polypeptide were unaffected when the Ca^{2+} chelator EDTA or when the calpain inhibitor calpeptin were included in the assay. These data corroborate that the HEK-293 cell line possesses basal calpain activity (Shimada *et al.*, 2005), and suggest that the wild-type $\text{Ca}_v\beta_3$ subunit is a target of this protease.

We next sought to determine whether the PEST regions found in $\text{Ca}_v\beta_3$ were substrates of calpain. To this end, proteolysis experiments of wild-type and mutant $\text{Ca}_v\beta_3$ proteins were conducted according to a protocol described elsewhere (Shumway *et al.*, 1999). Briefly, calpain activity was indirectly determined by assessing the extent of Ca^{2+} -induced degradation of $\text{Ca}_v\beta_3$ $\Delta\text{P1-2}$ mutant subunits compared to the wild-type protein degradation (Fig. 2C). Inhibitors such as EDTA and calpeptin were used in the assay to ascertain the specificity of calpain activity. As described above, incubation with Ca^{2+} (750 μM) caused degradation of $\text{Ca}_v\beta_3$ which was prevented in the presence of EDTA or calpeptin. On the other hand, examination of the $\text{Ca}_v\beta_3$ $\Delta\text{P1-2}$ mutant by Western blot evidenced that this protein does not undergo endogenous Ca^{2+} /calpain-induced proteolytic cleavage, illustrating the importance of the PEST sequences (Fig. 2C).

As a more direct test of the contributing role of the PEST domains as calpain substrates, we examined the proteolytic profile of the $\text{Ca}_v\beta_3$ subunit mutants in which the PEST sequences had been removed. First, $\text{Ca}_v\beta_3$ was produced by *in vitro* translation in the rabbit reticulocyte lysate, then mixed with increasing concentrations of calpain in the presence of Ca^{2+} to activate

the protease. The results indicated that the *in vitro* translated $\text{Ca}_v\beta_3$ protein is indeed susceptible to exogenous calpain breakdown in a dose- and Ca^{2+} -dependent manner. Notably, calpain concentrations of ~10 nM were sufficient to significantly degrade the *in vitro* translated $\text{Ca}_v\beta_3$, and this effect was prevented when EDTA (consistent with the Ca^{2+} requirement of calpain) or the calpain inhibitor calpeptin were present. Of note, these experiments were performed adding exogenous calpain, in contrast to those in which the protein is degraded showing two additional bands (where proteolysis was mediated by endogenous Ca^{2+} dependent proteases; Fig. 2A). We next questioned whether either the N- or the C-terminal PEST domains of $\text{Ca}_v\beta_3$ were molecular determinants for the efficiency of *in vivo* degradation by calpain. Recombinant $\text{Ca}_v\beta_3$ proteins lacking amino acid residues 24 to 37 (ΔP1) and 397 to 411 ($\Delta\text{P1-2}$) expressed in HEK-293 cells were subjected to increasing concentrations of μ -calpain and probed by Western blot. Though at a concentration of 90 nM (in the presence of 750 μM Ca^{2+}), the $\Delta\text{P1-2}$ mutant proteins were almost completely degraded, proteolytic breakdown of these proteins seemed to be reduced in comparison to the full-length $\text{Ca}_v\beta_3$ at equal concentrations of calpain (not shown). Despite the fact that the internal concentration is unknown, we surmise that a low concentration of the protease, in conjunction with the over-expression of our protein, explains that the effect is only partial. In contrast, when exogenous calpain is added, the proteolysis seems to be more potent. These results suggest that the PEST sequences within the $\text{Ca}_v\beta_3$ subunit promote its degradation by μ -calpain. In line with this, when the double PEST deletion mutant protein ($\Delta\text{P1-2}$) was incubated with calpain, we detected a significant decline in proteolysis relative to that of the single mutants. Following incubation with 50 nM calpain some of the input protein still remained (Fig. 2D). Taken together, these data suggest that deletion of the PEST sequences produced

$\text{Ca}_v\beta_3$ subunits that were more resistant to cleavage by calpain than the full-length protein and provide evidence that the extent of proteolysis depends on the presence of the PEST sequences.

In order to exclude the possibility that the reduced proteolytic sensitivity shown by the $\Delta\text{P1-2}$ mutant may be the result of gross structural alterations in the $\text{Ca}_v\beta_3$ protein caused by the amino acids deletion, we next tested whether the ΔPEST mutated versions of $\text{Ca}_v\beta_3$ could directly associate with the $\text{Ca}_v\alpha_1$ subunit through the Alpha1 Interaction Domain (AID), the main channel structure involved in the $\text{Ca}_v\alpha_1$ - $\text{Ca}_v\beta$ subunit interaction. To this end, we used a GST-fusion protein encoding a short fragment of the intracellular I-II loop of the $\text{Ca}_v2.2$ subunit carrying the AID region (GST-AID_{2.2}), and compared its binding to the *in vitro* synthesized [³⁵S]- β_3 wild-type and mutant proteins (Fig. 3). As can be seen, all of the [³⁵S]- $\text{Ca}_v\beta_3$ subunits maintained the ability to bind the GST-AID_{2.2} fusion protein indicating that the deletions in the PEST regions do not affect the association of these subunits to the purified GST fusion protein.

To extend these findings, we next investigated the functional repercussion of the PEST deletions in $\text{Ca}_v\beta_3$ by electrophysiological recording. The whole-cell mode of the patch clamp technique was used to study the macroscopic Ba^{2+} currents (I_{Ba}) through recombinant N-type Ca_v channels (composed of $\text{Ca}_v2.2$ and $\alpha_2\delta-1$) in HEK-293 cells transiently expressing wild-type $\text{Ca}_v\beta_3$ or its mutants. Figure 4A shows representative current traces recorded during depolarizing voltage steps to +10 mV from a holding potential of -80 mV. Control experiments carried out using cells transfected with the wild-type $\text{Ca}_v\beta_3$ showed that the average current density (peak current amplitude divided by the respective value of C_m) was -141 ± 24 pA/pF. Recordings performed in cells transfected with the PEST deletions revealed an up-regulation of the macroscopic Ba^{2+} current. As can be seen in Figure 4B, I_{Ba} density measured at +10 mV was significantly increased (~60%) in cells expressing the double PEST deletion ($\Delta\text{P1-2}$). Similar

results, with nearly the same level of I_{Ba} up-regulation, were also observed in cells expressing the single PEST deletions ($\Delta P1$ and $\Delta P2$).

The above described data are further illustrated in Figure 4C, which shows the I_{Ba} density as a function of the voltage step in transfected cells. 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. The stimulatory effects of PEST deletions on current densities were observed at almost all potentials explored. Interestingly, we noticed that PEST deletions also altered the macroscopic kinetic properties. Normalized currents obtained from either control or cells expressing $Ca_v\beta_3$ mutant subunits showed that the temporal course of the current traces was different (Fig. 4A). Though neither the time to peak nor the time constant for the activation of the current were apparently modified (data not shown), the time constant for the inactivation (τ_{inact}) and the percentage of current remaining after 140 ms activating pulses were significantly different between control and cells expressing the mutant subunits (Fig. 4D). It is worth mentioning that the use of Ba^{2+} as the charge carrier in these experiments may reduce Ca^{2+} -dependent inactivation. Together, these results suggest that the PEST sequences may be important for determining also the effects of $Ca_v\beta$ subunits on channel voltage-dependent inactivation properties. Alternatively, it is also possible that kinetic modifications may be induced by PEST1 deletion through a non specific structural alterations of the $Ca_v\beta_3$ subunit. On the other hand, the PEST2 region is less likely to be involved in such a non specific effect since previous studies have demonstrated that the third variable region (V3) of the protein (where PEST2 is located) may not be necessary for inactivation (Wittmann *et al.*, 2000; Opatowsky *et al.*, 2003).

Given that the deletion of the PEST sequences in $Ca_v\beta_3$ resulted in mutant proteins more resistant to calpain cleavage (Fig. 2) that enhanced functional expression levels of Ca_v channels

(Fig. 4C), we speculated that the calpain system may be responsible for the regulated degradation of the $\text{Ca}_v\beta_3$ subunit *in vivo*. Hence, the observed increase in current density through Ca_v channels containing mutant subunits may be the result of an increased stability of these proteins in intact cells. If this was the case, inhibition of calpain activity *in vivo* would decrease $\text{Ca}_v\beta_3$ wild-type turnover enhancing the availability of $\text{Ca}_v\beta_3$ and promoting the trafficking of the channels to the plasma membrane. Therefore, in order to see whether calpain degrades $\text{Ca}_v\beta_3$ under physiological conditions, we treated the HEK-293 cell line cultures with a specific calpain inhibitor. Traces in Figure 5A exemplify representative records of membrane currents through recombinant channels of the $\text{Ca}_v2.2/\alpha_2\delta$ -1 class co-expressing wild-type $\text{Ca}_v\beta_3$ obtained in untreated (control) HEK-293 cells and in cells exposed to calpeptin (25 μM). As can be seen, there was no significant change in whole cell I_{Ba} in cells treated for 3 h with the inhibitor. In contrast, longer exposure (6 h) to calpeptin resulted in a significant increase in I_{Ba} density (Fig. 5B). On the other hand, there was no significant effect on I_{Ba} density in HEK-293 cells expressing recombinant channels that included the $\text{Ca}_v\beta_3$ PEST mutant subunits after calpeptin treatment (Figs. 5C and D), consistent with the idea that calpain proteolysis affects $\text{Ca}_v\beta_3$ activity through the presence of PEST sequences. Taken as a whole, the results presented above suggest that calpain may be a physiological regulator of $\text{Ca}_v\beta_3$ protein turnover.

Lastly, as mentioned earlier, our data showed that the deletion of PEST like sequences decreases the degradation of $\text{Ca}_v\beta_3$ subunit *in vitro* (Fig. 2), we therefore aimed to reproduce these results *in vivo*. In order to determine whether the PEST region deletions affected protein stability, half-lives of the wild-type $\text{Ca}_v\beta_3$ subunit and its mutants were measured in metabolic pulse-chase experiments (Fig. 6). In these experiments, HEK-293 cells transiently transfected with the cDNA encoding the $\text{Ca}_v\beta_3$ subunits were labeled for 30 min with [^{35}S]-

methionine/cysteine and chased with unlabeled methionine and cysteine. After 24 h of chase, proteins were immunoprecipitated with a polyclonal anti- β_3 raised against the full-length $\text{Ca}_v\beta_3$ sequence to reveal most of the proteolytic fragments. As can be seen, the wild-type protein was synthesized as a band with a molecular mass of ~58 kDa which underwent progressive degradation (~85% was degraded after 24 h). This result is consistent with the idea that proteins containing PEST sequences usually have short half lives, and is also consistent with our finding that $\text{Ca}_v\beta_3$ may be the target of proteolytic breakdown under physiological conditions. With the ΔPEST mutants, however, less degradation occurred during the 24 h of chase (~28, ~32 and 45% for $\Delta\text{P1-2}$, ΔP1 and ΔP2 , respectively). This finding suggests that the elevated surface expression of the channels containing the $\text{Ca}_v\beta_3$ mutant proteins may be related with enhanced protein half-life.

Discussion

A number of proteins related to the metabolism or functions of intracellular Ca^{2+} have been reported to be substrates for calpain including Ca_v channels. Initially, the demonstration that the $\text{Ca}_v\alpha_1$ subunit was sensitive to calpain suggested a possible mechanism for regulation of Ca_v channel function. In the skeletal muscle, the C-terminal domain of the $\text{Ca}_v\alpha_{1.1}$ subunit is sensitive to proteolysis by μ -calpain (De Jongh *et al.*, 1994). This proteolytic cleavage is thought to remove a major site of phosphorylation providing a mechanism for modifying the cAMP-dependent regulation of L-type Ca^{2+} channels. In addition, calpain proteolysis has been shown to affect the functional activity of Ca_v channels. In patch clamp studies, Ca^{2+} currents decline progressively due to “rundown” which depends on the intracellular Ca^{2+} concentration. This led to the proposal that Ca_v channels could be degraded by a Ca^{2+} -dependent protease. Interestingly, Ca^{2+} current rundown in myocytes has been shown to be accelerated by calpain and retarded by the physiological calpain inhibitor calpastatin, suggesting that these proteins may be involved in the regulation of channel activity and/or turnover (Belles *et al.*, 1988; Romanin *et al.*, 1991).

At first glance, the first PEST region which is highly conserved across species and $\text{Ca}_v\beta$ subunits appears of particular interest. The last few amino acids of this region contribute to an alpha helix that precedes the beta strands of the SH3 domain in several crystal structures of $\text{Ca}_v\beta_{2a}$ (Opatowsky *et al.*, 2004; Van Petegem *et al.*, 2004). Hence, if conserved sequence mediates conserved function, the findings for $\text{Ca}_v\beta_3$ might be generalized to the entire family of $\text{Ca}_v\beta$ subunits. In addition, as we documented in the Results section, both PEST regions showed modulatory effects on the functional expression of neuronal recombinant Ca_v channels.

In the present work, we show that calpain proteolysis may also affect the $\text{Ca}_v\beta_3$ auxiliary subunit, providing a novel mechanism for modifying the regulation of Ca_v channels. As

mentioned earlier, it has been suggested that calpain may cleave proteins near regions containing PEST sequences (Rechsteiner and Rogers, 1996). It is proposed that these regions increase the local Ca^{2+} concentration and, in turn, activate calpain. Analysis of the $\text{Ca}_v\beta_3$ sequence using the PEST-Find computer program revealed two PEST-like domains in the protein (Fig. 1A). Notably, though the presence of the PEST regions in the sequences of the $\text{Ca}_v\beta$ subunits and their possible roles in subunit degradation was suggested initially several years ago (Ruth *et al.*, 1989; Perez-Reyes *et al.*, 1992), their physiological relevance remain virtually unexplored. It is worth mentioning that proteins containing PEST sequences typically have short half lives (~2 h) in intact cells compared with most other proteins (>24 hours). In these proteins, removal or disruption of the PEST sequence increases the half life of the protein while insertion or creation of a new PEST sequence within a PEST sequence free protein decreases this half life. Interestingly, it has been shown that the recombinant $\text{Ca}_v\beta_3$ subunit, when expressed alone in a mammalian cell line, is rapidly turned over (2-6 h) (Bogdanov *et al.*, 2000). In contrast to what we observed for the recombinant $\text{Ca}_v\beta_3$ used in this study (Fig. 6), which is consistent with a rapid turnover of the protein, it has been noted that the half-life of native $\text{Ca}_v\beta$ subunits is about 50 h (Berrow *et al.*, 1995). Though the reason for this discrepancy is unknown, it is possible that the association of the native $\text{Ca}_v\beta$ subunits with membrane bound proteins increases their stability.

It is worth mentioning also that proteolytic cleavage within a PEST sequence may not serve for protein degradation only. In this regard, an exciting possibility is that proteolytical cleavage of the full length $\text{Ca}_v\beta_3$ may result in the generation of short forms of the protein with potential physiological actions. This is particularly important after the identification of several novel fully functional short variants of the $\text{Ca}_v\beta_1$ and $\text{Ca}_v\beta_2$ subunits (Foell *et al.*, 2004; Harry *et*

al., 2004; Cohen *et al.*, 2005). In this context, our work might suggest that in addition to the splice isoforms generated through the genetically encoded deletions of specific regions in the $\text{Ca}_v\beta$ gene, smaller functional variants could be also formed by post-transcriptional processing of the protein.

On the other hand, the increase in Ca^{2+} current amplitude induced by transfection of $\text{Ca}_v\beta_3$ mutant subunits lacking the PEST regions in the HEK-293 cells suggest that the ΔPEST mutants are more stable than the wild-type protein. In this scenario, the availability of $\text{Ca}_v\beta_3$ would be enhanced which may reverse the inhibition imposed by the endoplasmic reticulum (ER) retention signal to the $\text{Ca}_v\alpha_1$ subunit facilitating the cell surface expression of the Ca_v channel complex. Indeed, PEST deletion seemed to make the ΔPEST mutant proteins less susceptible to calpain cleavage (Fig. 2).

In addition, expression studies have shown that gating as well as regulation of high voltage-activated Ca_v channels is for a large part determined by the interaction between the $\text{Ca}_v\alpha_1$ and the $\text{Ca}_v\beta$ subunits. Though a range of functional effects has been identified for $\text{Ca}_v\beta$, one of the most important actions of this protein is to facilitate the trafficking of the $\text{Ca}_v\alpha_1$ subunit to the plasma membrane, partly by its ability to mask the ER retention signal in $\text{Ca}_v\alpha_1$ (Bichet *et al.*, 2000). However, the $\text{Ca}_v\beta$ subunits can also affect the biophysical properties of Ca_v channels by changing the rates of activation and deactivation by voltage as well as by altering the rate of voltage-induced inactivation, the inhibition by G protein $\beta\gamma$ dimers, and/or the coupling of voltage sensing to pore opening (Birnbaumer *et al.*, 1998; Walker and De Waard, 1998; Arikath and Campbell, 2003; Dolphin, 2003).

In particular, diverse studies have revealed that $\text{Ca}_v\beta$ subunits have a marked effect on voltage-dependent inactivation of Ca_v channels, a key mechanism that contributes to the precise

control of Ca^{2+} entry into cells. Whilst the $\text{Ca}_v\alpha_1$ subunit contains inherent determinants of inactivation, association with different $\text{Ca}_v\beta$ subunits determines their overall inactivation rate (Birnbaumer *et al.*, 1998; Walker and De Waard, 1998; Dolphin, 2003). Though the precise mechanisms of voltage-dependent inactivation are not well understood, it is clear that subunit composition differentially affects the inactivation properties of Ca_v channels. In general, whole-cell patch clamp studies indicate that co-expression of $\text{Ca}_v\beta_{1b}$, β_{2a} , and β_4 with $\text{Ca}_v\alpha_1$ subunits do not modify the inactivation rate noticeably, whereas $\text{Ca}_v\beta_3$ markedly enhances inactivation (Dolphin, 2003). Interestingly, our functional studies on the effects of the PEST sequences using $\text{Ca}_v\beta_3$ mutant subunits suggested a role for these regions in the regulation of neuronal N-type recombinant Ca_v channel activity by inactivation (Fig. 4D).

In response to membrane depolarization, control Ca_v channels quickly activate followed by rapid inactivation (Fig. 4A). In contrast, the channels containing mutant $\text{Ca}_v\beta_3$ subunits lacking the PEST regions displayed slower inactivation kinetics, resulting in a much smaller fraction of inactivated channels at the end of the test pulse (Fig. 4D). In each case, the half-activation potentials closely aligned with those observed in the presence of the wild-type $\text{Ca}_v\beta_3$ subunit, and therefore, any putative effects of the mutant subunits on inactivation kinetics would unlikely be due to altered voltage dependence of activation gating. Interestingly, a variable region (V1) found at the N-terminal of $\text{Ca}_v\beta_3$ comprised of a short 14-amino acid stretch has been recently reported as a critical site for voltage-induced inactivation (Stotz *et al.*, 2004). In addition, a second variable region (V2) in combination with one of the conserved domains of the protein can also contribute to regulate $\text{Ca}_v2.2$ inactivation (Stotz *et al.*, 2004). However, the PEST1 sequence in $\text{Ca}_v\beta_3$ is not located in a variable region of the protein, suggesting the presence of numerous domains in $\text{Ca}_v\beta_3$ capable of conferring rapid inactivation kinetics.

Although diverse studies with $\text{Ca}_v\beta$ subunits suggest that some of their effects on channel activity require phosphorylation (Dolphin, 2003) little is known regarding the role of this process on Ca_v channel inactivation. By using the NetPhos 2.0 software (available at the URL <http://www.cbs.dtu.dk/services/NetPhos/>) which produces predictions for serine, threonine and tyrosine phosphorylation sites in eukaryotic proteins (Blom *et al.*, 1999), we found numerous phosphorylation sites for protein kinases on the $\text{Ca}_v\beta_3$ PEST-like sequences. Proteolysis of $\text{Ca}_v\beta_3$ by calpain would remove the phosphorylation consensus sites and the potential regulation of Ca_v channel inactivation through phosphorylation at these sites would also be abolished. Further studies are necessary to define whether the protein kinase-mediated regulation of the $\text{Ca}_v\beta_3$ subunit may be directly involved in the regulation of the inactivation process.

The biochemical and electrophysiological studies described in the present study show that the $\text{Ca}_v\beta_3$ auxiliary subunit is sensitive to μ -calpain digestion within its PEST-like regions and suggest that this enzyme may play a critical role in regulating $\text{Ca}_v\beta_3$ turnover. In addition, the results provide evidence that the $\text{Ca}_v\beta_3$ PEST-like sequences might be regulatory segments that influence the voltage-dependent inactivation properties of Ca_v channels. Lastly, if conserved sequence mediates conserved function, it would be interesting to investigate whether the findings for $\text{Ca}_v\beta_3$ could be generalized to the entire family of $\text{Ca}_v\beta$ subunits.

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FIGURE LEGENDS

Fig. 1. Identification and deletion of the PEST-like sequences in the $\text{Ca}_v\beta_3$ subunit. *A*, Schematic representation of the functional domains of $\text{Ca}_v\beta_3$ and the putative PEST sequences. Two potential PEST regions with scores of +9.47 (residues 24-37) and +11.52 (residues 397-411) were found in the amino acid sequence of $\text{Ca}_v\beta_3$ when analyzed with the PESTFind software. SH3 denotes a type 3 src-homology and GK-like indicate a guanylate kinase domain. *B*, Autoradiogram of *in vitro* translated [^{35}S]-methionine-labeled wild-type (β_3) and PEST deletion mutants of the $\text{Ca}_v\beta_3$ subunit (ΔP1 , ΔP2 , $\Delta\text{P1-2}$) resolved by SDS-PAGE. 5 μl of each translation reaction were run per lane. *C*, Western blot analysis of membranes from untransfected HEK-293 cells (-) or cells expressing the wild-type (β_3) and the $\text{Ca}_v\beta_3$ PEST deletion mutants (ΔP1 , ΔP2 , $\Delta\text{P1-2}$).

Fig. 2. The $\text{Ca}_v\beta_3$ subunit is cleaved by Ca^{2+} -dependent proteases. *A*, Recombinant $\text{Ca}_v\beta_3$ $\Delta\text{P1-2}$ protein is more stable than the wild-type $\text{Ca}_v\beta_3$ to endogenous Ca^{2+} -dependent proteases. *Lanes 1* and *2* show the proteolytic breakdown of wild-type $\text{Ca}_v\beta_3$ (β_3) heterologously expressed in HEK-293 cells. 200 μg of microsomes were incubated with Ca^{2+} (750 μM) at 30°C for 20 min in absence or presence of EDTA (1.5 mM). No proteolytic degradation was observed (lanes 3-4) when the PEST-like regions were deleted ($\Delta\text{P1-2}$). *B*, Coomassie-stained SDS-PAGE in a 10% resolving gel using Laemmli buffer system. *Lane 1*, control; *lanes 2-7*, microsomes from HEK-293 cells expressing the wild-type $\text{Ca}_v\beta_3$ incubated for 20 min at 30°C with Ca^{2+} , EDTA or calpeptin or exposed to increasing concentrations of μ -calpain. *C*, Degradation of the $\text{Ca}_v\beta_3$ subunits in the presence of Ca^{2+} and a calpain protease inhibitor. $\text{Ca}_v\beta_3$ subunits were incubated at 30°C for 20 min with 750 μM Ca^{2+} , 180 nM of calpeptin or 1.5 mM EDTA. The full-length

(β_3) or the double PEST deletion mutant ($\Delta P1-2$) are indicated. *D*, Δ PEST mutant $\text{Ca}_v\beta_3$ proteins were incubated for 20 min at 30°C with Ca^{2+} , EDTA or calpeptin as indicated above or exposed to two increasing doses of μ -calpain as listed (in the presence of 750 μM Ca^{2+}). In each case, one representative of at least two independent degradation experiments of wild-type and mutant $\text{Ca}_v\beta_3$ proteins is presented.

Fig. 3. Deletion of the PEST-like sequences did not alter the specific binding of the mutant $\text{Ca}_v\beta_3$ to the $\text{Ca}_v\alpha_1$ subunit. Determination of $\text{Ca}_v\beta_3$ binding to the AID of the $\text{Ca}_v2.2$ subunit. Capacity of the wild-type and mutant [^{35}S]- $\text{Ca}_v\beta_3$ subunits to interact with the fusion protein GST-AID_{2.2} was assayed by SDS-PAGE and autoradiography. Translation represents the equivalent volume of *in vitro* translation of [^{35}S]- $\text{Ca}_v\beta_3$ used in the binding assays.

Fig. 4. Deletion of the PEST-like sequences alters whole-cell I_{Ba} in HEK-293 cells. *A*, superimposed representative I_{Ba} recordings obtained from HEK-293 cells co-expressing neuronal recombinant $\text{Ca}_v2.2/\alpha_2\delta-1$ channels and the wild-type $\text{Ca}_v\beta_3$ subunit (control) or its PEST deletion mutants ($\Delta P1$, $\Delta P2$ and $\Delta P1-2$) in response to 140 ms test pulses to +10 mV from the holding potential of -80 mV. *B*, Comparison of peak I_{Ba} densities in $\text{Ca}_v2.2/\alpha_2\delta-1$ channels co-expressed with wild-type $\text{Ca}_v\beta_3$ or the PEST deficient mutants. Data are expressed as mean \pm S.E., and the number of recorded cells is indicated in parentheses. Statistical significance was determined by Student's *t*-test (*, $P < 0.05$). *C*, I_{Ba} density-voltage relationship averages for HEK-293 cells co-expressing neuronal recombinant $\text{Ca}_v2.2/\alpha_2\delta-1$ channels and the wild-type $\text{Ca}_v\beta_3$ subunit (control; *filled circles*, $n = 6$) or its single ($\Delta P1$; *open circles*, $n = 7$; and $\Delta P2$ *gray circles*, $n = 6$) and double PEST deletion mutants ($\Delta P1-2$; *filled squares*, $n = 7$). Currents were elicited by

eleven 140 ms depolarizing pulses between -40 and +60 mV in 10 mV increments from a holding potential of -80 mV. *D*, Comparison of inactivation time constants (τ_{inact} ; *gray bars*) and percentage of inactivated channels at the end of a test pulse to +10 mV (*open bars*) for I_{Ba} through $\text{Ca}_v2.2/\alpha_2\delta$ -1 channels co-expressing the wild-type $\text{Ca}_v\beta_3$ subunit or its ΔPEST mutants. The time course of inactivation was typically best fit with a monoexponential function. Bars represent mean \pm S.E. values of 25 cells in each condition. Statistically significant results are shown by the *asterisk* (*t* test; $P < 0.05$).

Fig. 5. Changes in the functional expression of recombinant N-type ($\text{Ca}_v2.2/\alpha_2\delta$ -1/ β_3) Ca^{2+} channels in HEK-293 cells treated with the specific calpain inhibitor calpeptin. *A*, Superimposed I_{Ba} traces recorded in untreated (control) cells expressing $\text{Ca}_v2.2/\alpha_2\delta$ -1/ β_3 channels and cells exposed to calpeptin (25 μM at 37°C for 3-6 h). The currents were elicited by 140 ms voltage steps to +10 mV from a holding potential of -80 mV. *B*, Summary histogram of I_{Ba} densities obtained from cells after 3 or 6 h of exposure to calpeptin. Densities were calculated on dividing peak current amplitudes elicited from voltage steps of -80 to +10 mV, by the whole-cell capacitance. *C*, Representative superimposed I_{Ba} traces in HEK-293 cells co-expressing $\text{Ca}_v2.2/\alpha_2\delta$ -1 and the mutant $\Delta\text{P1-2 } \beta_3$ subunit recorded as in *A*. *D*, Histogram of I_{Ba} densities obtained from calpeptin-treated cells as listed. Data are expressed as mean \pm S.E., and the number of recorded cells is indicated in parentheses. Statistically significant results are shown by the *asterisk* (*t* test; $P < 0.05$).

Fig. 6. The ΔPEST mutations have an increased protein half-life. Wild type $\text{Ca}_v\beta_3$ subunit and its ΔPEST mutants half-lives were determined in HEK-293 cells 24 h after transfection. Cells were

pulse-labeled for 40 min. at 37°C with 500 $\mu\text{Ci}/\mu\text{l}$ [^{35}S]-L-containing media. After the pulse, radioactive media was replaced by complete medium; cells were lysed after a 24 h chase period in complete media, and analyzed by SDS-PAGE. An immunoprecipitation with a polyclonal anti- β_3 IgG was performed to purify $\text{Ca}_v\beta_3$ variants and their proteolytic fragments. Lane 1 shows the control precipitation (incubation of protein A sepharose beads with protein extracts containing [^{35}S]-wild-type $\text{Ca}_v\beta_3$). Lanes 2, 3, 4 and 5 represent the endogenous proteolytic degradation of the wild-type $\text{Ca}_v\beta_3$ subunit and its PEST mutations ΔP1 , ΔP2 and $\Delta\text{P1-2}$ after immunoprecipitation by the anti- β_3 IgGs.