Supplementary Methods

Cloning of cDNA constructs

The integrity of all constructs was confirmed by DNA sequencing (MWG Biotech, Martinsried, Germany). In all constructs numbering refers to Genebank accession number NM_000720 and AJ224874 (length of open reading frame 5898 bp) for Ca\(_{\alpha\,1.3}\) \(\alpha1\) and Ca\(_{\alpha\,1.4}\) \(\alpha1\) subunits, respectively. Numbers refer to the amino acid (aa) or nucleotide positions (nt). Asterisks (*) indicate artificial restriction sites introduced by PCR.

The CSNB2 truncation mutant Ca\(_{\alpha\,1.4}\) K1951X was constructed by inserting a stop mutation at aa position 1591 followed by a NotI restriction site introduced by PCR. The resulting EcoRI (4348)-NotI*-cut PCR fragment was ligated into the EcoRI-NotI (nt 4348-6097)-cut Ca\(_{\alpha\,1.4}\) \(\alpha1\) \(pClneo\) cDNA. Deletion mutant Ca\(_{\alpha\,1.4}\) c122 was constructed by introducing a stop mutation at position aa 1845 followed by a SalI restriction site. The resulting EcoRI (nt 4348)-SalI* PCR fragment was co-ligated with NheI-EcoRI (nt 1-4348)-cut Ca\(_{\alpha\,1.4}\) \(pClneo\)-polyA into \(pClneo\) (Promega) in a three fragment ligation step.

Chimera Ca\(_{\alpha\,1.3}\)-C1.4, Ca\(_{\alpha\,1.3}\)-C1.4-122 and Ca\(_{\alpha\,1.3}\)-C1.4-203 were composed of aa 1-1620 of Ca\(_{\alpha\,1.3}\) \(\alpha1\) assembled with either the whole Ca\(_{\alpha\,1.4}\) \(\alpha1\) C-terminal fragment (aa 1567-1966), or parts of the C-terminus: Ca\(_{\alpha\,1.3}\)-C1.4-122 (aa 1567-1844) and Ca\(_{\alpha\,1.3}\)-C1.4-203 (aa 1567-1763). Corresponding sequences are shown as supplementary Figure 1. Chimeras were constructed by introducing stop codons at positions aa 1967, 1845 and 1764, respectively, followed by a BglIII restriction site. BstEII (4698)-BglIII* cut Ca\(_{\alpha\,1.4}\) \(\alpha1\) PCR fragments were assembled into BstEII-BamHI (4765-6427)-cut Ca\(_{\alpha\,1.3}\) \(\alpha1\) \(pGFP\) cDNA. Wildtype calmodulin (CaM) and dominant-negative CaM\(_{\alpha\,1.3}\) were a kind gift of J. Adelman.

For FRET analysis all C-terminal Ca\(_{\alpha\,1.4}\) \(\alpha1\) fragments were cloned either into the mammalian expression vector \(pEYFP-C1\) (Clontech) for N-terminal YFP-labelling or vector \(pECFP-N1\) (Clontech) for C-terminal CFP-labelling. For all N-terminally YFP-labeled
constructs 5'-HindIII and 3'-SalI restriction sites flanking nucleotide sequences corresponding to the following Ca,1.4 α1 aa positions were introduced (together with a C-terminal HA-tag) and cloned into HindIII-SalI-cut vector pEYFP-C1: fragment "EF-preIQ-IQ-postIQ": aa 1434-1844; fragment "EF-preIQ-IQ": aa 1434-1603; fragment "postIQ": aa 1604-1844; fragment "EF": aa 1434-1487; fragment "preIQ-IQ-postIQ": aa 1489-1844; "Ca,1.4-C-terminus": aa 1434-1966. To construct C122-CFP, 5'-EcoRI and 3'-BamHI restriction sites were introduced flanking nucleotide sequences corresponding to Ca,1.4 aa positions 1845-1966 and cloned into EcoRI-BamHI-cut vector pECFP-N1. Preparation of pcDNA3-CFP-CaM and pcDNA3-CFP-CaM1234 encoding for N-terminally CFP-tagged CaM and CaM1234, respectively, were performed as described.

Electrophysiological recordings

Whole-cell patch-clamp recordings in transiently transfected HEK-293 (tsA-201) cells were performed as described previously for wildtype and mutant Ca,1.4 channels using either 15 mM Ba\(^{2+}\) or Ca\(^{2+}\) as charge carrier. Recording solutions were composed as follows (in mM): intracellular solution: 135 CsCl, 10 Cs-EGTA, 1 MgCl\(_2\) adjusted to pH 7.4 with CsOH; bath solution: 15 BaCl\(_2\), 10 HEPES, 150 choline-Cl, and 1 MgCl\(_2\), adjusted to pH 7.4 with CsOH. Cells were recorded 2-3 days after transfection. Transfected cells were visualized as co-transfected protein fluorescence (GFP, or GFP- or YFP-labeled constructs co-expressed with the channel complex as indicated). Current-voltage (I-V) relationships were obtained by holding cells at -90 mV before applying 50 ms pulses to various test potentials. I-V-curves were fitted with the equation $I = G_{\text{max}}(V-V_{\text{rev}})/\{1 + \exp[(V_{0.5,\text{act}}-V)/k]\}$, where $V_{\text{rev}}$ is the extrapolated reversal potential, $V$ is the test potential, $I$ is the peak current amplitude, $G_{\text{max}}$ is the maximum slope conductance, $V_{0.5,\text{act}}$ is the half maximal activation voltage and $k$ is the slope factor. Percentage of inactivation was determined during specified time points of
depolarizing pulses from a holding potential of -90 mV to the peak current potential ($V_{\text{max}}$) of the $I-V$ relation of the individual cell. The voltage dependence of inactivation was assessed by holding cells at various holding potentials for 10 sec before application of a test depolarization to $V_{\text{max}}$. Inactivation curves were fitted with the Boltzman relationship $I = I_{SS} + (1 - I_{SS}) / (1 + \exp (V - V_{0.5,\text{inact}}/k_{\text{inact}}))$, where $I$ is the peak current amplitude, $I_{SS}$ is the noninactivating fraction, $V$ is the membrane potential, $V_{0.5,\text{inact}}$ is the half-inactivation potential, and $k_{\text{inact}}$ is the slope factor.

All voltages were corrected for liquid junction potential. CDI was quantified as the fraction of peak current remaining at the end of a 50-ms or 250-ms depolarization ($r_{50}$, $r_{250}$) as a function of test potential and by the parameter $f$, defined as the maximal difference between $r_{50}$ or $r_{250}$ values of $I_{Ba}$ versus $I_{Ca}$.

References

1. Koschak, A., Reimer, D., Huber, I., Grabner, M., Glossmann, H., Engel, J., & Striessnig, J. $\alpha 1$ (Ca$_{1.3}$) subunits can form L-type Ca$^{2+}$ channels activating at negative voltages. J. Biol. Chem. 276, 22100-22106 (2001).


