Structural determinants of Ca\textsubscript{v}1.3 L-type calcium channel gating

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**Abbreviations:** aa, amino acid; CDI, calcium dependent inactivation; CTM, C-terminal modulatory domain; LTCC, L-type calcium channel; Po, open probability; scg, superior cervical ganglion; \(V_{0.5}\), voltage of half maximal activation

A C-terminal modulatory domain (CTM) tightly regulates the biophysical properties of Ca\textsubscript{v}1.3 L-type Ca\textsuperscript{2+} channels, in particular the voltage dependence of activation (\(V_{0.5}\)) and Ca\textsuperscript{2+} dependent inactivation (CDI). A functional CTM is present in the long C-terminus of human and mouse Ca\textsubscript{v}1.3 (Ca\textsubscript{v}1.3\textunderscore L), but not in a rat long cDNA clone isolated from superior cervical ganglia neurons (rCa\textsubscript{v}1.3\textunderscore scg). We therefore addressed the question if this represents a species-difference and compared the biophysical properties of rCa\textsubscript{v}1.3\textunderscore scg with a rat cDNA isolated from rat pancreas (rCa\textsubscript{v}1.3\textunderscore q). When expressed in tsA-201 cells under identical experimental conditions rCa\textsubscript{v}1.3\textunderscore scg exhibited Ca\textsuperscript{2+} current properties indistinguishable from human and mouse Ca\textsubscript{v}1.3\textunderscore L, compatible with the presence of a functional CTM. In contrast, rCa\textsubscript{v}1.3\textunderscore scg showed gating properties similar to human short splice variants lacking a CTM. rCa\textsubscript{v}1.3\textunderscore scg differs from rCa\textsubscript{v}1.3\textunderscore L at three single amino acid (aa) positions, one alternative spliced exon (exon31), and a N-terminal polymethionine stretch with two additional lysines. Two aa (S244, A2075) in rCa\textsubscript{v}1.3\textunderscore scg explained most of the functional differences to rCa\textsubscript{v}1.3\textunderscore L. Their mutation to the corresponding residues in rCa\textsubscript{v}1.3\textunderscore L (G244, V2075) revealed that both contributed to the more negative \(V_{0.5}\), but caused opposite effects on CDI. A2075 (located within a region forming the CTM) additionally permitted higher channel open probability. The cooperative action in the double-mutant restored gating properties similar to rCa\textsubscript{v}1.3\textunderscore L. We found no evidence for transcripts containing one of the single rCa\textsubscript{v}1.3\textunderscore scg mutations in rat superior cervical ganglion preparations. However, the rCa\textsubscript{v}1.3\textunderscore scg variant provided interesting insight into the structural machinery involved in Ca\textsubscript{v}1.3 gating.

### Introduction

Voltage-gated Ca\textsubscript{v}1.3 Ca\textsuperscript{2+} channels represent a member of the dihydropyridine-sensitive class of L-type Ca\textsuperscript{2+} channels (LTCCs). Together with Ca\textsubscript{v}1.2 LTCCs they are expressed in mammalian electrically excitable tissues. Ca\textsubscript{v}1.3 knockout mice\textsuperscript{1-3} and a recently discovered human disease (Sinoatrial Node Dysfunction and Deafness, SANDD)\textsuperscript{4} revealed a key role of these channels for hearing, cardiac pacemaker function and neuronal excitability. Ca\textsubscript{v}1.3 channels possess unique gating properties that privilege them to carry Ca\textsuperscript{2+} inward currents at threshold potentials in sinoatrial node cells,\textsuperscript{5} chromaffin cells\textsuperscript{6} neurons as well as within the operating range of cochlear inner hair cells.\textsuperscript{6,7} These gating properties are tightly controlled by a C-terminal modulatory domain (CTM) with strong effects on Ca\textsubscript{v}1.3 activation and inactivation gating behavior as well as open probability (Po).\textsuperscript{8,10} The CTM is formed by an intermolecular protein-protein interaction of a distal (DCRD) with a proximal C-terminal modulatory domain (PCRD), thereby decreasing the calmodulin affinity for the channel.\textsuperscript{11,12} Alternative splicing within the Ca\textsubscript{v}1.3 C-terminus is tissue-specific and involves structural motifs required for high affinity interaction with calmodulin and the proper function of the CTM. Since calmodulin serves as the Ca\textsuperscript{2+} sensor for autoinhibitory Ca\textsuperscript{2+}-dependent inactivation (CDI) of voltage-gated Ca\textsuperscript{2+} channels, splicing causes pronounced gating differences between long and short Ca\textsubscript{v}1.3.\textsuperscript{8,9,11-13,34} Short CTM-deficient splice variants have higher Po, activate at more negative voltages and show accelerated CDI than full length variants with a functional CTM (Ca\textsubscript{v}1.3\textunderscore L).\textsuperscript{8,9} This modulatory domain has been extensively characterized with cDNA constructs cloned from human pancreas,\textsuperscript{8,9} and its effects were also reproduced with short and long mouse constructs (unpublished observations). In contrast, the modulation was not reported for Ca\textsubscript{v}1.3 channels cloned from rat superior cervical ganglia neurons (scg) (termed rCa\textsubscript{v}1.3\textunderscore scg).\textsuperscript{15} Despite the presence of a full length C-terminal tail, rCa\textsubscript{v}1.3\textunderscore scg showed gating properties similar to those observed in short splice variants,\textsuperscript{11,15-20} suggesting a dysfunctional CTM. It is at present unclear if this could represent a significant species difference in Ca\textsubscript{v}1.3 channel modulation and to which extent transcripts corresponding to rCa\textsubscript{v}1.3\textunderscore scg are expressed in rat scg (and perhaps other tissues). Despite considerable use of rCa\textsubscript{v}1.3\textunderscore scg constructs in Ca\textsubscript{v}1.3 research,\textsuperscript{11,13-18} it has never been tested if...
corresponding transcripts indeed exist. Moreover, the molecular determinants responsible for the observed functional differences between rCa\textsubscript{1.3\_scg} and other rat and mammalian Ca\textsubscript{1.3\_L} α\textsubscript{1}\textsubscript{-}subunits have never been systematically analyzed although they could provide novel information about structural features controlling channel gating. Here we provide bioinformatic and biochemical evidence that rCa\textsubscript{1.3\_scg} transcripts are indeed not expressed at detectable levels in rat scg and should therefore not be considered to be contributing to Ca\textsubscript{1.3} functional diversity. We took advantage of the minor aa differences between rCa\textsubscript{1.3\_scg} and the long rCa\textsubscript{1.3} α\textsubscript{1}\textsubscript{-}subunit (rCa\textsubscript{1.3\_L}) predicted from the rat cacna1d gene. This allowed us to identify these residues as those responsible for the abnormal gating behavior of rCa\textsubscript{1.3\_scg} and as key elements in Ca\textsubscript{1.3} Ca\textsuperscript{2+} channel gating.

**Results**

Major gating differences between rCa\textsubscript{1.3\_scg} and rCa\textsubscript{1.3\_L}. We have recently discovered that an intramolecular protein interaction within the C-terminus of long Ca\textsubscript{1.3} α\textsubscript{1}-subunit splice variants forms CTM that stabilizes a gating mode with activation at a more positive voltage range, slower CDI and lower Po. When analyzed under identical experimental conditions after expression in tsA-201 cells, we found that this regulation is present in the long variant of human Ca\textsubscript{1.3} (Ca\textsubscript{1.3\_L})\textsuperscript{9} and the common rat rCa\textsubscript{1.3\_L} α\textsubscript{1}\textsubscript{-}subunit originally isolated from rat pancreas (termed rCa\textsubscript{1.3\_L} in analogy to the human Ca\textsubscript{1.3\_L} containing a full length C-terminal tail\textsuperscript{8}) but not in the corresponding long rat rCa\textsubscript{1.3} variant isolated from scg (rCa\textsubscript{1.3\_scg}). The activation threshold and V\textsubscript{0.5} of rCa\textsubscript{1.3\_scg} was about 16 mV more negative (Fig. 1A and Table 1) and the inactivation time course of Ca\textsuperscript{2+} currents (I\textsubscript{Ca}) was significantly faster than for rCa\textsubscript{1.3\_L} (Fig. 1B and Table 2) and human Ca\textsubscript{1.3\_L} (not shown, cf. 8 and 9). Faster inactivation was due to enhanced CDI as evident from the even slower inactivation when equimolar Ba\textsuperscript{2+} served as the charge carrier (I\textsubscript{Ba}, Fig. 1B). This finding indicates potential species differences or the existence of a scg-specific Cav1.3 splice variant. We therefore investigated the expression of the rCa\textsubscript{1.3\_scg} variant in rat scg. rCav1.3scg transcripts were not detected in rat scg RNA preparations. Comparison of the aa sequence of the rCa\textsubscript{1.3\_scg} α\textsubscript{1} construct with the canonical rat Ca\textsubscript{1.3\_L} α\textsubscript{1} sequence revealed only minor structural differences (Fig. 2A). Single aa exchanges were found in positions 244 (S in rCa\textsubscript{1.3\_scg}, G in rCa\textsubscript{1.3\_L}), 1104 (V to A) and 2075 (A to V), an alternatively spliced locus (exon 31 in rCav1.3 scg vs. exon 31A in rCa\textsubscript{1.3\_L}) also found in mouse and human,\textsuperscript{21} and a polymethylene stretch with two additional lysines at the N-terminus (7M2K), which is present in the mRNA of different species but was absent in the rCav1.3 scg construct.\textsuperscript{15} Inspection of the rat genomic cacna1d-sequence (GenBank accession number NW_047469.2) revealed the DNA sequence previously reported for rCav1.3 L α\textsubscript{1}\textsubscript{-}subunits, isolated from pancreas (D38101, D38102), brain (M57682) and kidney (M99221). We found no genomic evidence for any of the single amino acid differences reported for rCav1.3 scg. The residues concerned are also highly conserved in other species, such as human (EU363339.1), mouse (NP_001077085), cattle (NP_001179954) and rabbit (XP_002713437), with no evidence for variation as found in rCav1.3 scg. Since the three single aa differences S244, V1104, A2075 had been detected in scg preparations\textsuperscript{15} but not in rat brain,\textsuperscript{22} we re-evaluated the abundance of these variants in Sprague-Dawley scg preparations, by using a transcript-scanning approach. rCa\textsubscript{1.3\_scg} and rCa\textsubscript{1.3\_L}-specific sequences were determined by restriction enzyme profiling of cloned PCR products. Neither S244 (n = 122) nor V1104 (n = 106) or A2075 (n = 109) could be detected (they would appear as undigested PCR products in lanes b, and digested PCR products in lanes c in Fig. 2B) in more than hundred clones analyzed for each position.

**Figure 1.** Gating differences between rCa\textsubscript{1.3\_scg} and rCa\textsubscript{1.3\_L}. (A) Normalized voltage activation range of rCa\textsubscript{1.3\_scg} (○) and rCa\textsubscript{1.3\_L} (●). Activation parameters are given in Table 1. Error bars are often smaller than symbol size. (B) Inactivation of I\textsubscript{Ca} (scg, L) and I\textsubscript{Ba} (scg Ba\textsuperscript{2+}, L Ba\textsuperscript{2+}) of rCa\textsubscript{1.3\_scg} and rCa\textsubscript{1.3\_L} during the first 500 ms of a 2.5 sec test pulse to V\textsubscript{max}. Inactivation parameters obtained by fitting the data to a double exponential function are given in Table 2. Data are shown as means (black line) with SE (gray). (C) Representative experiments, described in (B) and corresponding fits are illustrated.
**Table 1. Biophysical properties of rCav1.3 α1,-subunit mutations**

| Ca1.3 construct | $V_{ax}$ | act thresh | $k_{ax}$ | $V_{rev}$ | n |
|-----------------|---------|-----------|---------|---------|---|
| rCav1.3scg      | -21.34 ± 0.98 | -50.30 ± 0.89 | 8.51 ± 0.30 | 63.38 ± 1.07 | 23 |
| rCav1.3L        | -5.39 ± 0.73*** | -39.43 ± 0.51*** | 9.10 ± 0.12 | 65.37 ± 0.62 | 55 |
| rCav1.3scg;S244G| -11.20 ± 0.78** | -43.47 ± 0.64** | 8.86 ± 0.17 | 65.22 ± 1.15 | 30 |
| rCav1.3scg;V1104A| -14.00 ± 1.29 | -47.51 ± 1.00 | 9.05 ± 0.26 | 65.25 ± 1.53 | 7 |
| rCav1.3scg;A2075V| -11.66 ± 0.91 | -44.15 ± 1.39 | 8.88 ± 0.24 | 62.87 ± 0.88 | 11 |
| rCav1.3scg;S244G| -10.23 ± 0.88 | -42.59 ± 0.89 | 8.75 ± 0.27 | 63.93 ± 1.14 | 12 |
| rCav1.3scg;V1104A/A2075V| -8.87 ± 0.87 | -40.99 ± 0.63 | 8.70 ± 0.15 | 64.21 ± 0.78 | 14 |
| rCav1.3scg;7M2K/S244G/A2075V| -5.66 ± 0.99*** | -37.10 ± 0.92*** | 7.94 ± 0.31 | 69.29 ± 0.77 | 11 |

Parameters (means ± S.E.) were obtained by fitting data of I–V relationships as described in methods; statistical significances are indicated for comparisons vs. rCav1.3scg (a–aaa), vs. A2075V (b–bbb) and vs. S244G/A2075V (c, mL, ccc) (one-way Anova with Bonferroni post-test).

This suggests minimal or no rCav1.3scg expression in adult rat scg. Direct sequencing of PCR products amplified from two independent reverse transcriptions also revealed no evidence for low abundance DNA sequence variations in the codons concerned (Fig. 2C). Therefore our data do not confirm the expression of rCav1.3scg-specific sequences in scg.

As shown above we found profound functional differences between rCav1.3scg and rCav1.3L. Therefore the observed aa differences provided us with a unique opportunity to study their role for Ca1.3 channel function.

**Impact of single and combined mutations on rCav1.3scg gating.** To reveal the contribution of these minor structural differences for channel gating we first converted the single aa exchanges individually (S244G, V1104A, A2075V) and in combination to rCav1.3 residues within the rCav1.3scg backbone and tested to which extent this resulted in restoration of rCav1.3 functional properties. We also analyzed the alternatively spliced locus (exon 31/exon 31A) and the N-terminal polymethionine stretch with two additional lysines (7M2K). S244 in rCav1.3scg is located at the cytoplasmatic end of segment S4, which forms part of the voltage sensing domain in repeat I (Fig. 2A). Conversion of S244 to the corresponding glycine in rCav1.3 (S244G) shifted the activation threshold (by 6.03 ± 1.47 mV, p < 0.001) and $V_{V_{05}}$ (3.06 ± 1.59 mV) to more positive voltages (Fig. 3A and Table 1). It also induced a significant decrease in $k_{ax}$ (Table 1). S244G enhanced inactivation of $I_{ca}$ during depolarizations to test potentials over a broad voltage range (Fig. 3B and Table 2). Since no increase in inactivation was observed for $I_{ax}$ (Fig. 3B) this enhancement reflected accelerated CDI. Faster inactivation resulted from a significant decrease of the time constant of the fast inactivating component ($\tau_{ax}$) and a significant increase in its contribution to inactivation (Fig. 4 and Table 2). As an estimate for changes in channel Po we compared the relative size of ON gating currents ($Q_{on}$) with tail current amplitude ($I_{tail}$). This ratio was similar for rCav1.3scg and S244G suggesting that the mutation did not change Po (Fig. 5). Taken together, mutation S244G in rCav1.3scg partially restores the more positive activation threshold of rCav1.3L, but accelerates CDI. Among a large number of point mutations previously studied,23,24 S244G is to our knowledge the first mutation capable of significantly enhancing CDI. This finding was surprising because the mutation was expected to reduce CDI to the more moderate level observed in rCav1.3L.

In contrast to S244G, reverting A2075 in rCav1.3scg to the corresponding valine in rCav1.3L (A2075V) inhibited CDI (Figs. 3B and 4). Quantification over a large voltage range revealed that CDI was strongly decreased and even significantly slower than in rCav1.3L (see F-values in Table 1). Voltage-dependent inactivation (i.e., inactivation kinetics of $I_{ax}$, VDI) was not affected (Fig. 3B). Slowing of CDI by the A2075V mutation resulted in a significant about 8-fold increase in $\tau_{ax}$ and a 22% decrease of the contribution of the fast component (Fig. 4 and Table 2). Like S244G, A2075V also caused a partial but significant shift of $V_{ax}$ to more positive voltages by about 6 mV (Fig. 3A and Table 1). These data show that although both single mutations shift the channel’s activation voltage range to more positive voltages, they exert opposite effects on CDI. Also in contrast to S244G, A2075V increased $Q_{on}$ relative to $I_{tail}$ (Fig. 5) suggesting a decrease in Po. Mutation of V1104 in rCav1.3scg to alanine, or inclusion of the 7M2K sequence at the N-terminus (Tables 1 and 2) caused no significant effects on $V_{ax}$, activation thresholds and on current kinetics. We therefore hypothesized that the combined mutation of S244G and A2075V in rCav1.3scg restores most of the gating properties of rCav1.3L. In the double mutant S244G/A2075V CDI parameters were restored to values indistinguishable from those of rCav1.3L (Figs. 3 and 4). This was again verified over a broad voltage range showing that F-values of S244G/A2075V and rCav1.3 were not significantly different (Fig. 3B). VDI was also not affected by the double mutation (Fig. 3B). Introduction of V1104A into A2075V (V1104A/A2075V) was without noticeable effect on gating parameters (Tables 1 and 2).

The double mutation S244G/A2075V robustly shifted $V_{ax}$ by about 10 mV to more positive voltages, thus representing...
an additive effect of the two individual mutations (Table 1). However, it still activated at about 6 mV more negative voltages than rCa\(_{1.3}\)\(_{\text{scg}}\) (Fig. 3A and Table 1). To further determine the structural basis of this remaining difference we constructed a variety of combined mutations. Inclusion of V1104 and exon31A in S244G/A2075V neither alone nor in combination restored the \(V_{\text{0.5}}\) of rCa\(_{1.3}\)\(_{\text{L}}\) (Table 1). However, the additional insertion of the rCa\(_{1.3}\)\(_{\text{L}}\) N-terminal peptide 7M2K (that completely restored the sequence of rCa\(_{1.3}\), although in a different expression plasmid\(^{25}\)) restored the voltage activation range of rCa\(_{1.3}\)\(_{\text{L}}\) as expected. This indicates a complex cooperative interaction between 7M2K, exon31 and A1104 for control of activation gating.

**Discussion**

The recent discovery of a C-terminal modulatory domain within the long C-terminal tail of LTCCs provided a fresh perspective on how these channels undergo automodulatory fine tuning through C-terminal intramolecular protein-protein interaction. In Ca\(_{1.4}\)\(_{\alpha}-\)subunits this interaction can completely suppress CDI. This stabilizes very slow inactivation of Ca\(^{2+}\) currents as required for continuous Ca\(_{1.4}\) channel activity in retinal photoreceptor terminals.\(^{26-28}\) In Ca\(_{1.2}\) this modulatory domain serves as the structural framework to enable its regulation by cAMP-dependent protein kinase during the flight-and-fight response.\(^{29,30}\) Ca\(_{1.3}\) variants with full-length or truncated C-termini show pronounced differences in gating behavior that affect Ca\(_{1.3}\)\(_{\text{mediated Ca}^{2+}}\) entry during physiological neuron-like firing patterns.\(^{8}\) Therefore the isolation of rat scg Ca\(_{1.3}\) channel transcripts (rCa\(_{1.3}\)\(_{\text{scg}}\), GenBank accession numbers: AF370010, AF370009) containing specific single amino acid differences and completely lacking this regulatory capacity\(^{11,15}\) implied a puzzling species difference. As the exchanges are not predicted by the genomic cacna1d sequence (GenBank accession number: NW_047469.2), they could result from nuclear pre-mRNA editing by adenosine
deaminases (ADARs). This is the most abundant type of RNA editing found in higher eukaryotes and has been reported for Ca1.3 α-subunits.10,31 It results in a post-transcriptional single nucleotide change from adenosine to inosine, which is interpreted as guanosine by the translation machinery. As evident from Figure 2C, however, this mechanism cannot explain the single amino acid changes in rCa1.3,scg. Our PCR analysis of reverse transcribed RNA samples from rat scg could not detect the single amino acid differences specifically reported for rCa1.3,scg (GenBank accession numbers: AF370010, AF370009). We therefore conclude that, if rCa1.3,scg transcripts exist at all, they are of very low abundance. However, we cannot rule out the possibility that rCa1.3,scg is the result of molecular processing not yet described for Ca1.3 α-subunits (such as utilization of microexons32) that occurs conditionally (e.g., in an age or health state-dependent manner) and has thus evaded our analysis.

We show that the long rCa1.3 α-subunit splice variant isolated from rat pancreas exhibits gating properties indistinguishable from those cloned from human and mouse. We found that the mutation of two aa residues in rCa1.3,scg to rCa1.3,sequence also restored most of the gating properties of rCa1.3,scg. Both mutations additively contributed in shifting V0.5 to more positive voltages. However, their effects on CDI were more complex. Whereas S244G enhanced CDI beyond inactivation of rCa1.3,scg, A2075V caused a strong reduction of CDI that was even slower than for rCa1.3,. These opposite effects eventually led to inactivation of S244G/A2075V very similar to rat and human Ca1.3., which were not different. These data also suggest that Ca1.3 constructs containing either serine in position 244 or alanine in position 2075, but not both, would display unusual biophysical properties different from all other Ca1.3 splice variants described so far. However, the presence of both induced gating properties very similar to human and mouse short Ca1.3.

The cytoplasmic side of the S4 transmembrane voltage-sensor helices transmits voltage-dependent movements to their S4-S5 linkers which make contacts to the pore-forming S6 helices and thereby participate in channel opening and closing.33 Accordingly, mutations in this region affect channel gating as, for example, seen in mutations associated with human diseases. Mutation S229P in Cav1.4 (causing CSNB2) completely prevents channel opening23 and S218L in Ca1.2 (causing Familial Hemiplegic Migraine Type I) causes a strong negative shift in V0.5 and a slowing of VDI.35 It is unknown how S244G could enhance CDI even beyond the fast CDI already seen in rCa1.3,scg. In addition, how can we explain the observation that mutations S244G and A2075V both cause a similar positive shift of V0.5 but show opposite effects on CDI? One possible unifying interpretation is based on a recent model assuming an allosteric CDI mechanism24 in which inactivation represents an allosteric modulation of channel opening. It assumes that channels can adopt either a normal gating mode or an “inactivated” mode in which their opening is allosterically inhibited but not completely prevented by interaction with calmodulin. In this model mutations that promote opening would also enhance opening of the inactivated mode and therefore diminish CDI (i.e., CDI observed when all channels are in the inactivated mode). Conversely, mutations diminishing opening (i.e., increasing V0.5) could enhance CDI. However, with increasing V0.5 also the fraction of channels in the inactivated mode at steady-state (FCDI) would decrease, due to diminished Ca2+ entry to drive CDI. This would result in a bell-shaped relationship between relative changes in V0.5 and observed CDI. However, mutations that strongly suppress maximum Po would also reduce FCDI that resulted in observed CDI values to fall below the predicted bell-shaped curve. Therefore we measured the relationship of Q gating currents to Itail as an estimate for changes in maximum Po.32,24 We found no changes for S244G but a strong reduction of maximal Po for A2075V. Accordingly, the increase in CDI induced by S244G can be explained by the more positive V0.5 in the absence of changes in Po. In contrast, diminished CDI of A2075V must be determined by its lower maximal Po. Moreover, A2075V is known to decrease the affinity of the C-terminal regulatory domain for the apo-calmodulin binding site on the channel11 which must also be regarded as a strong inhibitory factor for CDI.

![Figure 3. Voltage-dependent activation of rCa1.3, mutants.](image-url)
We conclude that rCav1.3.scg should not be regarded as contributor to the previously described functional heterogeneity of Cav1.3 channels as long as no unequivocal bioinformatic and biochemical evidence indicates significant expression in scg neurons or other tissues. Note that at present no evidence exists for any species differences with regard to Cav1.3 channel gating. As Cav1.3 channels are currently a promising target for drug discovery to develop neuroprotective agents in Parkinson disease,3,37 the complex stabilization of gating properties by opposite effects of two single amino acid changes should be taken into account when interpreting previous studies employing rCav1.3.scg constructs on the modulation of channel currents by protein interactions,16,38 enzymes,39 or mutations.24

**Methods**

**RNA preparation.** Five male Sprague-Dawley rats (10–11 weeks old) were euthanized by carbon dioxide inhalation in a saturated chamber in accordance with established welfare guidelines. The superior cervical ganglia (scg) were excised and stored in liquid nitrogen immediately. Total RNA was purified from pooled tissue using the Qiagen RNeasy Lipid Tissue Kit according to manufacturer's protocol. RNA concentration was measured using Nanodrop (NanoDrop 1000 Spectrophotometer, Thermo Scientific), and RNA quality was evaluated via separation of 28S and 18S rRNA bands on a denaturing agarose gel. One microgram of total RNA was reverse transcribed at 55°C (RevertAid™ Premium First Strand cDNA Synthesis Kit, Fermentas) using random hexamer primers in a reaction volume of 20 μl. The concentration of the cDNA yielded was referred to in RNA equivalents, i.e., 1 μl of reverse transcription reaction (cDNA) was equivalent to 50 ng of RNA.

**Transcript scanning and restriction analysis.** PCR reactions were performed with Pfu DNA Polymerase (EP0501, Fermentas)
and 50 ng RNA equivalent from independent reverse transcription (95°C for 2 min, 30 cycles of 95°C for 30 sec, 58°C for 30 sec, 72°C for 1 min 15 sec, final elongation at 72°C for 5 min; 0.5 μM primer, 2 mM MgSO₄, 0.2 mM dNTP). Specific primer pairs (position (pos) 244: Fwd, 5’-GGA CTG CTG CTG CAT CCT AAT GCT-3’; Rev, 5’-GGA GCT GGG TCC TCT TCA GCT AC-3’; pos 1104: Fwd 5’-TGC ACA GAT GAG GCC CCT AAT GCT-3’; Rev, 5’-CCT CTC GGC GAG CCT CTG GTT AA-3’; pos 2075: Fwd, 5’-GCC GTC GCT ACA CCG CAG TT-3’; Rev, 5’-CCT CTC GGC GAG CCT CTG GTT AA-3’; pos 1104: Fwd, 5’-TGC ACA GAT GAG GCC CCT AAT GCT-3’; Rev, 5’-CCT CTC GGC GAG CCT CTG GTT AA-3’), amplified fragments of 398 bp (pos 244), 620 bp (pos 1104) and 395 bp (pos 2075), respectively.

PCR products of independent PCR reactions, with independently generated cDNA templates, were purified by electrophoresis in 1.5% agarose gels and extracted using the NucleoSpin Kit (Macherey-Nagel) and ligated with the pJET1.2/blunt vector (Clone JET PCR Cloning Kit, Fermentas) for transformation of DH5α cells. Resulting clones were analyzed by colony PCR (95°C for 3 min, 30 cycles of 95°C for 30 sec, 60°C for 30 sec, 72°C for 45 sec, final elongation at 72°C for 5 min) using PCR Master Mix (K0171, Fermentas) primers flanking the multiple cloning site of the vector (Fwd, 5’-CGA CTC ACT ATA GGG AGA GCG GC-3’, Rev, 5’-AAG AAC ATC GAT TTT GGA CTG CTG CTG CAT-3’). One aliquot of the PCR reaction was used as undigested control; other aliquots were used for restriction enzyme analysis. For each amino acid position analyzed two randomly chosen clones were also verified by sequencing (Eurofins MWG Operon).

For all clones restriction analysis was performed with enzymes specifically digesting rCa v 1.3 - containing residues: Hpy188III for G244, Hin6I for A1104, Alw26I for V2075. As a control about 10% of clones were additionally analyzed with enzymes specifically digesting rCa v 1.3 specific-sequence: BstMI/MuI for S244, SduI for V1104, Bpu10I for A2075. Specific primers (see above) were used to generate positive control PCR fragments from cloned rCa v 1.3. Expected product sizes (bp) for samples were as follows: G244: a, 517; b, 217 + 152 + 63 + 50 + 35; c, 517; A1104: a, 739; b, 529 + 210; c, 739; V2075: a, 514; b, 261 + 253; c, 514. Expected digestion product sizes (bp) for controls: S244: 215 + 183; V1104: 468 + 152; A2075: 199 + 196 (Fig. 2B).

For direct PCR product sequencing gel extracted samples were directly sequenced (Eurofins MWG Operon) after PCR amplification from two independently generated scg cDNA templates and gel electrophoresis as described above.

Cell culture and transient expression. Human embryonic kindney cells (HEK293), stably expressing the SV40 temperature sensitive T antigen (tsA201), were grown in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% v/v fetal calf serum (10270-106, Gibco), 2 mM L-glutamine (25030-032, Gibco), and 10 units/ml penicillin (P-3032, Sigma)/streptomycin (S-6501, Sigma), maintained at 37°C in an 5% CO₂. Cells were split, when reached 80% confluency, using 0.05% Trypsin for cell dissociation. Passage did not exceed 20 numbers. Transient transfection was achieved by standard calcium phosphate precipitation method as described, using an equimolar ratio of cDNA encoding rCa v 1.3 - (generously provided by Diane Lipscombe), rCa v 1.3 - (generously provided by S. Seino) and the indicated mutants was determined from the ratios of maximal tail (ionic) current (I tail ) to Q on (ON gating current) amplitude. Data shown are means ± SE. In comparison to rCa v 1.3, Po was significantly changed for all constructs except for mutant S244G (one-way ANOVA with Bonferroni post-test). The insert shows representative current tracres for rCa v 1.3 - (black) and rCa v 1.3 - mutant A2075V (gray). Currents are normalized to maximal I tail amplitude. The experimental pulse protocol is shown above.

Figure 5.

Changes in maximal Po of rCa v 1.3 -mutants. Po of rCa v 1.3 - mutants 7M2K and A2075V were introduced into rCa v 1.3, rCa v 1.3 - and the indicated mutants was determined from the ratios of maximal tail (ionic) current (I tail ) to Q on (ON gating current) amplitude. Data shown are means ± SE. In comparison to rCa v 1.3, Po was significantly changed for all constructs except for mutant S244G (one-way ANOVA with Bonferroni post-test). The insert shows representative current tracres for rCa v 1.3 - (black) and rCa v 1.3 -mutant A2075V (gray). Currents are normalized to maximal I tail amplitude. The experimental pulse protocol is shown above.

Electrophysiology. For whole cell patch clamp recordings borosilicate glass electrodes where pulled (micropipette puller, Sutter Instruments) and fire polished (microforge, Narishige MF-830), having a final resistance of 2–5 MΩ. Cells were recorded in whole-cell configuration using an Axopatch 200B amplifier (Axon instruments). Data were digitized (Digitizer 1322A, Axon Instruments) and analysis was performed by pClamp 10.2 software (Axon Instruments). The recording solutions contained in mM: pipette solution: 135 CsCl, 10 HEPES, 10 Cs-EGTA, 1 MgCl₂, adjusted to pH 7.4 with CsOH; bath solution: 15 CaCl₂ or BaCl₂, 10 HEPES, 150 choline-Cl and 1 MgCl₂, adjusted to pH 7.4 with CsOH. Cells were held at a...
holding potential of -80 mV, before current-voltage (I-V) relationships were obtained by applying 10 ms square pulse protocol to various test potentials. For leak subtraction a P/4 protocol was used. I-V curves were fitted to the equation $I = G_{\text{max}}(V - V_{\text{rev}})/[1 + \exp((V - V_{\text{0.5}})/k_{\text{act}})]$, 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, $V_{\text{0.5}}$ is the half maximal activation voltage and $k_{\text{act}}$ is the slope factor. Inactivation was determined by a 2.5 sec long pulse to $V_{\text{rev}}$ and fit by standard double exponential decay, via Graph Pad Prism 5 (GraphPad Software Inc.). Voltage dependence of the inactivation time course was determined by a 300 ms long step protocol to various test potentials.

As an estimate for the maximal channel open probability we compared the amplitude of ON-gating currents ($Q_{\text{on}}$) with ionic tail currents ($I_{\text{tail}}$) during 10 ms test pulses to 61 mV and repolarization to -80 mV.

**Statistics.** Data analysis was performed by Clamplt 10.2 (Axon Instruments) and Sigma Plot 11 (Systat Software Inc.). All data are represented as mean ± SE. For statistical analysis one-way ANOVA with Bonferroni post-test or Student’s t-test was performed using Graph Pad Prism 5.1 software (GraphPad Software Inc.). Significance level was set to $p < 0.05$. 

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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