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Nuclear Localization and Dominant-negative Suppression by a Mutant SKCa3 N-terminal Channel Fragment Identified in a Patient with Schizophrenia*

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The small conductance calcium-activated K^+ channel gene *SKCa3/KCNN3* maps to 1q21, a region strongly linked to schizophrenia. Recently, a 4-base pair deletion in *SKCa3* was reported in a patient with schizophrenia, which truncates the protein at the end of the N-terminal cytoplasmic region (SKCa3Δ). We generated a green fluorescent protein-SKCa3 N-terminal construct (SKCa3–1/285) that is identical to SKCa3Δ except for the last two residues. Using confocal microscopy we demonstrate that SKCa3–1/285 localizes rapidly and exclusively to the nucleus of mammalian cells like several other pathogenic polyglutamine-containing proteins. This nuclear targeting is mediated in part by two polybasic sequences present at the C-terminal end of SKCa3–1/285. In contrast, full-length SKCa3, SKCa2, and IKCa1 polypeptides are all excluded from the nucleus and express as functional channels. When overexpressed in human Jurkat T cells, SKCa3–1/285 can suppress endogenous SKCa2 currents but not voltage-gated K^+ currents. This dominant-negative suppression is most likely mediated through the co-assembly of SKCa3–1/285 with native subunits and the formation of non-functional tetramers. The nuclear localization of SKCa3–1/285 may alter neuronal architecture, and its ability to dominantly suppress endogenous small conductance K_{Ca} currents may affect patterns of neuronal firing. Together, these two effects may play a part in the pathogenesis of schizophrenia and other neuropsychiatric disorders.

Ca^{2+} signaling cascades play a critical role in the functional activity of diverse tissues. Ca^{2+}-activated K^+ (K_{Ca})[1] channels modulate Ca^{2+}-mediated events by regulating the membrane potential in both excitable and non-excitable cells. K_{Ca} channels are divided into three groups (big, intermediate, and small) based on their single channel conductance (1). The small conductance SK_{Ca} channels have unitary conductances of 4–14 pS, are voltage-independent, and open in response to an increase in cytosolic $[Ca^{2+}]_i$ in the 200–500 nM range (2, 3). These channels are expressed in the central nervous system, where they modulate the firing pattern of neurons by generating slow membrane after-hyperpolarizations (3–5). Three genes (*SKCa1–3*) (6) with a conserved genomic organization (7) encode SK_{Ca} channels, their gene products bearing 70–80% amino acid sequence identity. Functional SK_{Ca} channels are formed from the homo- or heterotetrameric association of the SKCa1–3 subunits, each subunit containing six transmembrane helices (S1-S6) and intracellular N and C termini (8). Calcium-dependent gating of SK_{Ca} channels is mediated by calmodulin tightly complexed to the C terminus of each subunit (9, 10).

The human *SKCa3* gene (also known as *KCNN3* and *SK3*) has been implicated in schizophrenia (11), a psychiatric disorder that affects ~1% of all human populations. The gene maps to human chromosome 1q21 (12), a region containing a major susceptibility locus for some forms of familial schizophrenia (13). The SKCa3 channel contains a highly polymorphic polyglutamine repeat in its N terminus (11). Association studies on diverse human populations have shown that longer repeats are significantly over-represented in patients with schizophrenia (11, 12, 14–17), and one study (16) has further demonstrated that longer repeat alleles are associated with the negative symptoms of the disease. However, one family study reported over-representation of short alleles in schizophrenia (18), whereas several other association and family studies have failed to confirm any association between polyglutamine length in SKCa3 and schizophrenia (19–24). Recently, a rare 4-base pair deletion in the *SKCa3* gene was identified in a patient with schizophrenia (25). This frameshift mutation is predicted to generate a truncated SKCa3 polypeptide, SKCa3Δ, that ends prematurely at a stop codon after residue 286. ΔSKCa3 is identical to the SKCa3 sequence AF031815 up to Leu^{283} but then diverges replacing Ser^{284}, Asp^{285}, and Tyr^{286} in AF03815 with three frameshifted residues, Thr^{284}, Met^{285}, and Leu^{286}. The truncated protein spans the entire N terminus and lacks the hydrophobic core and C terminus. Interestingly, the SKCa3 N-terminal region contains two polybasic putative nuclear localizing signals (NLS), raising the possibility that the mutant protein might traffic to the nucleus as has been reported for several pathogenic alleles of other polyglutamine-repeat proteins (26–30), either alone or in combination with the product of the normal allele. Given the tetrameric nature of these channels, the mutant protein might also co-assemble with normal SK_{Ca} subunits encoded by all three loci in this gene family and suppress all endogenous SK_{Ca} currents in a dominant-

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[1] The abbreviations used are: K_{Ca}, calcium-activated K^+ channel, SK_{Ca}, small conductance K_{Ca}, GFP, green fluorescence protein, NLS, nuclear localizing signal; PCR, polymerase chain reaction; FCS, fetal calf serum; RBL, rat basophilic leukemic; HEK, human embryonic kidney; DMEM, Dulbecco's modified Eagle's medium.

negative manner. Such suppression has recently been demonstrated with an N-terminal SKCa2 fragment analogous to the truncated N-terminal SKCa3 protein (31).

In the present study we compared the intracellular trafficking of GFP-tagged full-length K_{Ca} proteins or a N-terminal SKCa3 protein (SKCa3–1/285) in mammalian cells and visualized the subcellular localization of these fusion proteins by confocal microscopy. Also, the dominant-negative effect of SKCa3–1/285 overexpression on endogenous SKCa2 currents in human Jurkat T cells was characterized by whole-cell patch clamp recording.

EXPERIMENTAL PROCEDURES

GFP Constructs—The GFP-tagged human *SKCa2*, *SKCa3*, and *IKCa1* cDNAs have been described (7, 31, 32). The C-terminal GFP-tagged constructs were generated in the *pEGFP-N*I expression vector (CLONTECH, Palo Alto, CA). SKCa3–1/285 and the deletions (SKCa3–1/270 and SKCa3–1/175) were generated using polymerase chain reaction (PCR) to engineer both a 5′ *Eco*RI site near the start codon and a 3′ *Bam*HI site at the appropriate position. These PCR fragments were cloned in-frame into *pEGFP-C1* (CLONTECH) to create two N-terminal GFP fusion constructs.

Cell Lines—All cell lines and media were purchased from ATCC, Manassas, VA and maintained according the ATCC protocols at 37 °C in 5% CO_2 and with 2 mM glutamine and 10% FCS (except as noted). Green monkey kidney (COS-7), rat basophilic leukemic (RBL) cells, human embryonic kidney (HEK), mouse pheochromocytoma (PC12), and mouse fibroblasts (L929) cells were cultured in DMEM, Jurkat T cells (E61) were grown in RPMI, and the human neuroblastoma (SK-N-SH) cell line was grown in DMEM with 20% FCS.

Transfection of Constructs into Mammalian Cells—Cells (5×10^5 cells/ring) were plated in culture chambers constructed by attaching a rubber ring to a glass coverslip with Sylgard and enclosing these rings in a sterile cell culture dish. Cells were transiently transfected with the GFP constructs and controls using the FuGene 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN) following the manufacturer's recommended protocol in OptiMEM media. Following an 8–12-h transfection, the cells were placed in DMEM supplemented with 10% FCS and 2 mM glutamine and incubated at 37 °C in 5% CO_2 for 24 h. The DNA vectors used for transfection were prepared using the Qiagen endotoxin-free Midi plasmid prep kit (Qiagen, Inc., Valencia, CA).

Confocal and Transmission Light Microscopy—Following transfection, confocal fluorescent images were taken with a Bio-Rad MRC-600 (Bio-Rad) equipped with an Argon laser (488 nm) and a fluorescein isothiocyanate filter set (500–530 nm). All images were acquired under a 63× oil immersion objective. The Scion image program (NIH, Bethesda, MD) was used to determine the pixel densities of nuclear and cytosolic fluorescence that are shown as figure insets.

Electrophysiology—To assess functional expression of ion channels, whole-cell recordings were carried out according to standard procedures (31).

RESULTS

SKCa3–1/285 Localizes in the Nucleus of Transfected Mammalian Cells—An alignment of the truncated SKCa3 protein SKCa3Δ sequence with that of SKCa1, SKCa2, and IKCa1 is shown in Fig. 1. SKCa3Δ is identical to AF031815 up to Leu^{283} and contains three additional frameshifted residues, Thr^{284}, Met^{285}, and Leu^{286} (25). The SKCa3 protein is unique in the SK_{Ca} channel subfamily in that it contains two polyglutamine-repeat cassettes separated by a proline-rich segment with six repeats of the putative SH3 binding motif P*XX*P. The remaining ~100 residues of SKCa3Δ contain two polybasic regions that may function as NLS motifs. The proximal polybasic motif NLS1 is present in SKCa3 and partially present in SKCa2, whereas the distal motif NLS2 is present in all four channels.

To determine whether SKCa3Δ can traffic to the nucleus, we generated a GFP-tagged N-terminal SKCa3 construct, GFP-SKCa3–1/285, that is identical to SKCa3Δ up to Leu^{283} but contains Ser^{284} and Asp^{285} from AF031815 in place of the three frameshifted residues in SKCa3Δ. SKCa3–1-285 was transfected into COS-7 cells, and the fluorescent fusion protein was

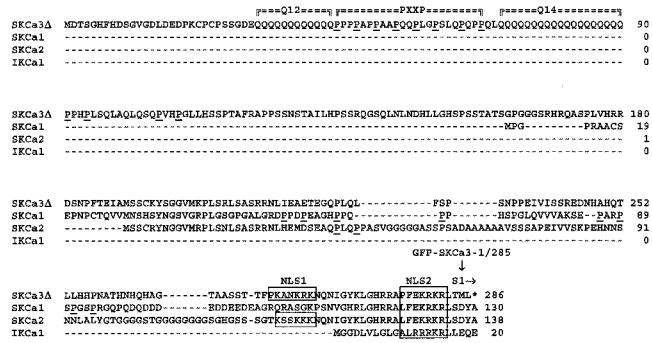

FIG. 1. **Alignment of the mutant SKCa3 protein sequence with that of SKCa1, SKCa2, and IKCa1.** SKCa3Δ (25), SKCa1 (accession number NM 002248), SKCa2 (AF239613), and IKCa1 (AF033021) are shown. The two polyglutamine repeats are labeled Q12 and Q14. The polyproline stretch with the PXXP motifs (*underlined*) and the two polybasic sequences (*boxed*) are highlighted. SKCa3 has two additional P*XX*P motifs just downstream of the second polyglutamine repeat (*underlined*). The three frameshifted residues in SKCa3Δ (Thr^{284}, Met^{285}, and Leu^{286}) are shown.

visualized by confocal microscopy. As controls, we evaluated the subcellular localization of the GFP-tagged full-length channels, SKCa3, SKCa2, and IKCa1. The three full-length channel constructs are excluded from the nucleus and are seen in presumptive endoplasmic reticulum and surface membranes (Fig. 2, *A–C*). Patch clamp studies have shown that these GFP-tagged channels function normally (7, 31, 32). In striking contrast, SKCa3–1/285 localizes exclusively in the nucleus of COS-7, regardless of whether the GFP tag is on the N-terminal (Fig. 2*D*) or C-terminal end (Fig. 3*A*). Nuclear targeting is rapid, occurring as early as 6 h after transfection (data not shown). Nuclear trafficking is also seen in the human Jurkat T cell line (Fig. 3*B*) and the human neuroblastoma line SK-N-SH (Fig. 3*C*), as well as in PC12, RBL, and L929 fibroblasts and HEK cells (data not shown). Deletion of the region containing the distal NLS2 motif (GFP-SKCa3–1/270) attenuates the exclusive nuclear staining, the fusion protein being seen in both the nucleus and cytoplasm (Fig. 3*D*). A further deletion that removes both NLS motifs (GFP-SKCa3–1/175) produces a similar labeling pattern with brightly staining nuclei, diffuse cytoplasmic labeling, and weak membrane staining (data not shown). This nuclear localization was unexpected, because an N-terminal SKCa2 fragment containing similar motifs was excluded from the nucleus (31). Taken together, these data confirm the hypothesis that SKCa3–1/285 traffics into the nucleus mediated in part by the region containing the distal NLS.

GFP-SKCa3–1/285 Suppresses Endogenous SK_{Ca} Currents—Given that functional SK_{Ca} channels are homo- or heterotetramers of SKCa1–3 subunits, SKCa3–1/285 might co-assemble with native SK_{Ca} subunits to produce non-functional tetramers. Consistent with this idea, a recent study demonstrated dominant-negative suppression of native SKCa2 channels in human Jurkat T cells transfected with an N-terminal SKCa2 fragment analogous to SKCa3–1/285 (31). Similar suppression of endogenous voltage-gated K^+ currents has been observed in cells transfected with K_V N-terminal fragments containing the structurally defined tetramerization domain (33).

To test whether SKCa3–1/285 can suppress endogenous currents, we transfected GFP-SKCa3–1/285 into Jurkat T cells that are known to express SKCa2 and Kv1.3 currents (34, 35). Patch clamp experiments were performed in control and GFP-SKCa3–1/285-transfected Jurkat cells. Fig. 4*A* shows a ramp protocol eliciting K^+ currents in human Jurkat T lymphocytes. The SKCa2 channels produce K^+ currents at potentials more negative than −40 mV, whereas at depolarized potentials voltage-gated Kv1.3 channels and SKCa2 channels both contribute

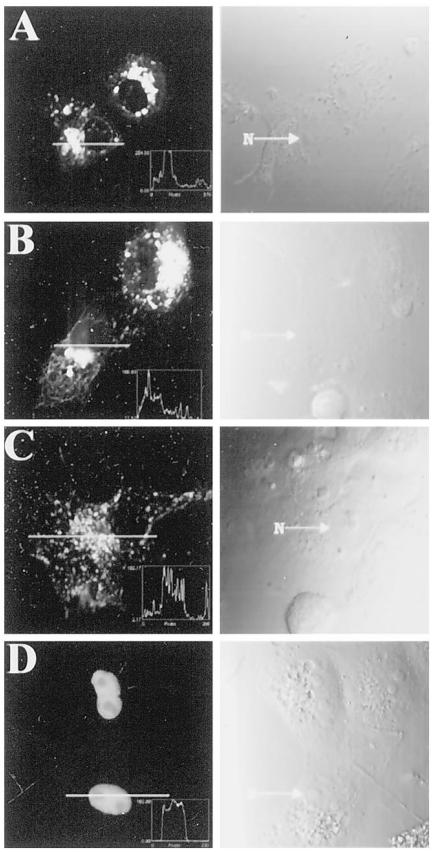

FIG. 2. **Confocal and transmission light images of COS-7 cells expressing K_{Ca} constructs.** *A*, GFP-SKCa3; *B*, GFP-SKCa2; *C*, GFP-IKCa1; and *D*, GFP-SKCa3–1/285. The *inset* in the confocal images shows the pixel density along the line shown. *N*, nucleus.

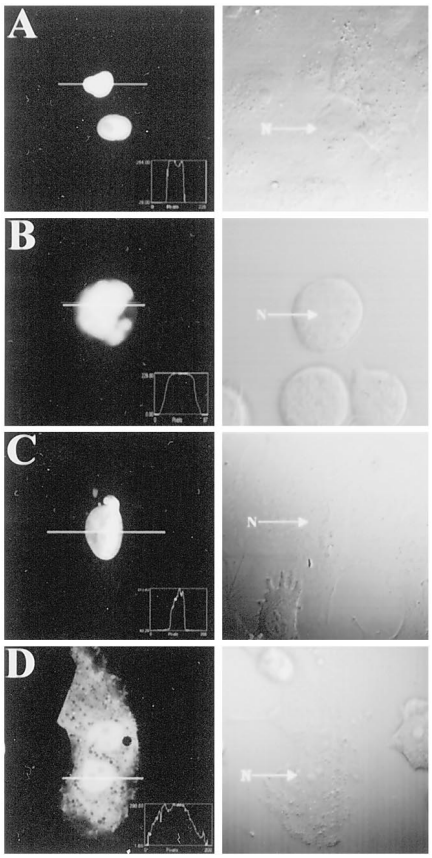

FIG. 3. **Subcellular localization of GFP-SKCa3–1/285 and GFP-SKCa3–1/270.** Confocal and transmission light images. *A*, SKCa3–1/285-GFP in COS-7 cells; *B* and *C*, GFP-SKCa3–1/285 in Jurkat and SK-N-SH, respectively; *D*, GFP-SKCa3–1/270 in COS-7 cells. The *inset* in the confocal images shows the pixel density along the line shown. *N*, nucleus.

current. The magnitude of the SKCa2 current increases at 40 mM $[K^+]_o$ *versus* 4.5 mM $[K^+]_o$. Expression of the truncated mutant SKCa3 protein in Jurkat T cells abolished whole-cell SKCa2 currents (Fig. 4*B*) compared with control cells, without affecting endogenous Kv1.3 currents. These results, taken together with earlier published data (31), indicate that SKCa3–1/285 can suppress endogenous SKCa2 currents via tetramerization with native subunits.

DISCUSSION

We have used confocal microscopy to demonstrate dramatic and exclusive nuclear localization of a truncated N-terminal SKCa3 protein, SKCa3–1/285, in mammalian cells. This construct, with the exception of two residues at the C terminus, is identical to SKCa3Δ, a mutant protein found in a patient with schizophrenia. Nuclear targeting is mediated in part by two polybasic NLS motifs, and this localization might have deleterious effects on neuronal architecture and/or function. Parallel patch clamp experiments show that SKCa3–1/285 can selectively suppress endogenous SKCa2 currents (Fig. 4), presumably by co-assembly with newly synthesized wild-type SK_{Ca} subunits producing non-functional tetramers. These results suggest a potent dominant-negative mechanism whereby SKCa3–1/285 may affect the function of the entire class of SK_{Ca} channels and thereby possibly alter brain function in schizophrenia.

SKCa3 transcripts are distributed in human and rat brain, including the substantia nigra, ventral tegmental area, caudate-putamen, amygdala, thalamus, medial habenula, lateral septal nucleus, dorsal raphe, hippocampus, and supraoptic nucleus (6, 12, 36–38). *SKCa1* and *SKCa2* transcripts have an overlapping pattern of expression and are also present in many

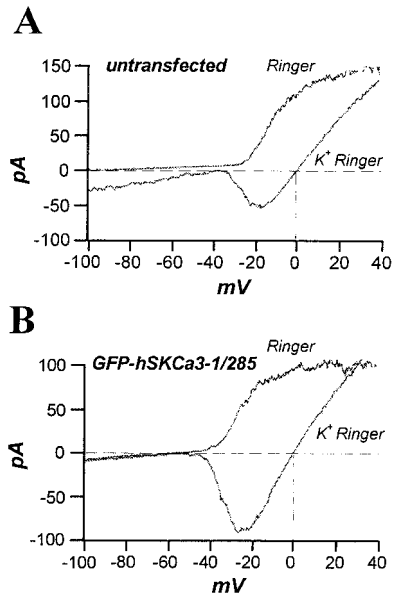

FIG. 4. **Suppression of endogenous SKCa2 currents in human Jurkat T cells by GFP-SKCa3–1/285.** *A*, endogenous SKCa2 and Kv1.3 currents in human Jurkat T cells at 4.5 and 40 mM $[K^+]_o$, respectively. *B*, suppression of SKCa2 but not Kv1.3 currents in cells transfected with GFP-SKCa3–1/285. Shown is a whole-cell recording from cells dialyzed with 1 μM Ca^{2+} in the pipette.

of these regions (36), suggesting that channel redundancy might mask even homozygous null mutations at any one locus. Dominant-negative suppression by SKCa3–1/285 could lead to

a global reduction of the entire family of SKCa1-SKCa3 channels in a fashion more potent than traditional allelic genetic suppression involving a single locus. Such reduction of all endogenous SK_{Ca} currents in regions of the brain, many of them rich in dopamine-containing neurons, might have effects analogous to the neurotoxic SK_{Ca} blocker apamin. Blockade of SK_{Ca} currents by apamin in brain slices is known to inhibit the post-spike afterhyperpolarization and change the firing pattern in dopamine-containing neurons from a pacemaker-like discharge into a multiple bursting pattern (39–41). In fact, recent studies demonstrate that SKCa3 is the intrinsic pacemaker of dopaminergic neurons in the substantia nigra (5). Bursting activity in turn has been associated with increased dopamine release (40–44). Apamin has also been reported to increase serotonin release in rat striatum (45). If dominant-negative suppression by SKCa3–1/285 has similar functional consequences, it might affect dopaminergic and serotonergic pathways in the brain that have been implicated in schizophrenia (46).

The nuclear localization of SKCa3–1/285 is reminiscent of the nuclear trafficking of other pathogenic polyglutamine proteins such as huntingtin (26–30). Although the two polyglutamine tracks in SKCa3–1/285 are shorter (12 and 14 Gln) than the critical threshold reported to be pathogenic for other proteins, a monoclonal antibody specific for expanded polyglutamine-containing proteins (>40 Q-long) has been shown to recognize the human SKCa3 protein (47). Thus, the tandem polyglutamine repeats and the intervening P*XX*P-containing polyproline segment in SKCa3–1/285 might mimic longer pathogenic polyglutamine repeats. Although the consequences of nuclear localization of SKCa3–1/285 are not yet known, any effect it has on the viability or architecture of neurons could be associated with the pathogenesis of schizophrenia. In keeping with this notion, postmortem and neuroimaging studies on schizophrenic brains have revealed a decrease in brain volume presumably because of a reduction of neuritic processes (48, 49). The presence of a putative proline-rich SH3-binding domain in the SKCa3 N terminus also raises the possibility that SKCa3–1/285 may bind to critical signaling molecules and potentially alter their subcellular localization and function.

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