Design and Characterization of a Highly Selective Peptide Inhibitor of the Small Conductance Calcium-activated K+ Channel, SkCa2

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Apamin-sensitive small conductance calcium-activated potassium channels (SKCa1–3) mediate the slow afterhyperpolarization in neurons, but the molecular identity of the channel has not been defined because of the lack of specific inhibitors. Here we describe the structure-based design of a selective inhibitor of SKCa2. Leiurotoxin I (Lei) and PO5, peptide toxins that share the RXCQ motif, potently blocked human SKCa2 and SKCa3 but not SKCa1, whereas maurotoxin, Pi1, Tsc, and PO1 were ineffective. Lei blocked these channels more potently than PO5 because of the presence of Ala1, Phe2, and Met7. By replacing Met7 in the RXCQ motif of Lei with the shorter, unnatural, positively charged diaminobutanoic acid (Dab), we generated Lei-Dab7, a selective SKCa2 inhibitor (Ki = 3.8 nM) that interacts with residues in the external vestibule of the channel. SKCa3 was rendered sensitive to Lei-Dab7 by replacing His521 with the corresponding SKCa2 residue (Asn367). Intracerebroventricular injection of Lei-Dab7 into mice resulted in no gross central nervous system toxicity at concentrations that specifically blocked SKCa2 homotetramers. Lei-Dab7 will be a useful tool to investigate the functional role of SKCa2 in mammalian tissues.

Ca2+-activated K+ (KCa) channels modulate cytosolic Ca2+ concentrations in excitable and non-excitable tissues by regulating the membrane potential. Based on their unitary conductance, KCa channels are classified as BKCa, IKCa, and SKCa (1). Opening in response to an increase in cytosolic [Ca2+]i in the 200–500 nM range (2), SKCa channels modulate the firing pattern of neurons by generating slow membrane afterhyperpolarizations (3–5). In the liver, they are believed to play a role in metabolic stress responses (6), and in human Jurkat T cells, they promote calcium entry in response to a mitogenic stimulus (7). SKCa channels have also been implicated in fasciculations in denervated skeletal muscle (8) and in myotonic dystrophy (9). SKCa channels, products of three phylogenetically related genes SKCa1–3, are found in a variety of tissues including the nervous system (10), liver (11), skeletal and smooth muscle (8, 12), adrenal medulla (13), and lymphoid tissues (14, 15). In the brain (16), the precise functional role of each channel in specific neuronal pathways has been difficult to elucidate because of the absence of selective blockers. The identity of the specific SKCa channel(s) responsible for apamin-induced destruction of cerebellar Purkinje neurons (17) and altered seizure threshold (18, 19) and for apamin and Lei neurotoxicity (20) is also unclear. The development of inhibitors that target each of these channels selectively would facilitate studies to determine their specific roles in mammalian tissues.

The SKCa2 channel is expressed in the brain (10, 16), retina (21), liver (22), melanocytes (expressed sequence tag AA418096), fetal heart (expressed sequence tag AA418000), and human Jurkat T cell line (7, 22, 23). Precise determination of function has only been possible in Jurkat T lymphocytes in which this channel plays a role in calcium signaling (7, 14, 24). The existing blockers of SKCa2 channels are not adequately specific to distinguish this channel from other SKCa subtypes. Apamin, a peptide from bee venom, exhibits only 10-fold selectivity for human SKCa2 over human SKCa1 or SKCa3 (3, 22, 25, 26), while the bisquinolinium cyclophane UCL-1684 blocks SKCa2 and SKCa1 with roughly equivalent potency (25) although it shows some selectivity over SKCa3 (7). The peptide toxin Lei (also known as scyllatoxin) from the scorpion Leiurus quinquestratius hebraeus, currently the most specific inhibitor of SKCa2, exhibits ~200-fold selectivity for human SKCa2 (15) over SKCa1 (25), although its affinity for SKCa3 had not been determined.

This study describes the guided design and electrophysiologic characterization of a novel Lei analog that selectively blocks SKCa2 homotetramers with low nanomolar affinity. Our strategy to design a specific inhibitor of the SKCa2 channel exploited an approach used previously to develop selective blockers of KvL3 and IKCa1 channels (27, 28). In this approach, a potent yet non-selective inhibitor of the target channel is the starting template. Specificity for the desired channel is engineered into the peptide on the basis of experimentally determined differences in its interaction with the target channel and other channels. Using Lei and a closely related peptide PO5 as templates, and by comparing the potencies of the native and mutant toxins for SKCa1, SKCa2, and SKCa3, we delineated the channel-binding surfaces of these toxins. Lei-Dab7 was...
designed to target a structural feature unique to the Lei-SKCa2 interaction surface.

**EXPERIMENTAL PROCEDURES**

**Peptide Synthesis**—N-α-Fmoc-t-amino acid derivatives, Fmoc-amide resin, and chemical reagents used for peptide synthesis were purchased from PerkinElmer Life Sciences (Shelton, CO), Novabiochem (Laufelfingen, Switzerland), and NeoSystem Laboratoire (Strasbourg, France). Solvents were analytical grade products from SDS (Peprin, France). The various peptides were synthesized by the stepwise solid-phase method (29) using a peptide synthesizer (Model 433A, Applied Biosystems Inc., Foster City, CA). The side-chain protecting groups used for trifunctional residues were: 2,2,5,7,8-pentamethylnorbornane-6-sulfonyl for Arg and homoarginine; tert-butylxycarbonyl for Orn, Lys, and homolysine, and 1-(4-dimethyl-2,6-dioxycyclohex-1-yliden)-3-methylbutyl for Dab and diaminopropionic acid (Dapa). The reduced peptides were dissolved at 1 mM in 0.2 M Tris-HCl buffer, pH 8.3, and stirred under air to allow folding/oxidation (48 h, 25 °C). The folded/oxidized toxins and their structural analogs were purified to homogeneity by reversed-phase high-pressure liquid chromatography (HPLC) (PerkinElmer Life Sciences), C18 Aquapore ODS 20 μm, 250 × 10 mm. The homogeneity (>99%) and identity of the peptides were verified by: (i) amino acid analysis, (ii) reversed-phase HPLC, (iii) amino acid composition determination after acetylation, and (iv) mass analysis by matrix-assisted laser desorption ionization-time of flight mass spectrometry.

**Cell Culture**—Jurkat E6–1, COS-7, and PC12 cells were obtained from ATCC (Manassas, VA). Jurkat E6–1 cells were grown in RPMI medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 10 mM HEPES, pH 7.4, 290 mOsm. K+V currents were recorded with a potassium aspartate-based pipette solution, SKCa2 currents were seen at negative potentials. Following break-in with 1 mM Ca2+ solution containing 50 nM free Ca2+, the inward rectifier (rKir 2.1) in RBL cells was studied in symmetrical Ca2+-free solutions to 80 mV applied every 30 s, and channel block measured at 80 mV by the toxin was taken as a measure of SKCa2 current. Following break-in with 1 mM Ca2+ solution, SKCa2 currents were amplified from human brain total RNA using reverse transcriptase polymerase chain reaction (PCR) with an engineered 5′ HindIII site near the start codon and a 3′ BamHI site near the termination codon and was cloned in-frame into the pEGFP-C3 vector (CLONTECH, Palo Alto, CA) to create GFP-SKCa1. PCR was used to generate mutant SKCa3 channels (28). PCR products were digested with KpnI and BamHI and cloned into KpnI and BamHI-cut GFP-SKCa3. All clones were verified by sequencing. DNA for transfection was prepared with the QIAGEN (Valencia, CA) Miniprep kit. Human SKCa2 in pcDNA3 was a generous gift from Dr. Bernard Attali (Tel Aviv University, Sackler School of Medicine, Israel).

**Transfection of Constructs into Mammalian Cells**—COS-7 cells were plated in culture chambers (5 × 105 cells/chamber), and 12–24 h later, cells were transiently transfected using FuGENE™ 6 (Roche Molecular Biochemicals) with the respective DNA in serum-free OptiMEM medium (Life Technologies, Inc.) as per the manufacturer’s recommended protocol. GFP-positive cells were used for electrophysiological studies at 48 h following transfection. Typical transfection efficiencies using this protocol were 40–70%. PC12 cells were plated overnight on glass coverslips prior to use. Cell lines stably expressing mKv1.1, mKv1.3, and RBL cells expressing endogenous rKir 2.1 were used for the selectivity screen as described previously (28).

**Electrophysiology**—Cells were studied in the whole cell configuration of the patch clamp technique. The holding potential in all experiments was −80 mV. For measurement of IKv,m, SKCa,m, and BK,Ca currents, we used an internal pipette solution containing (in mM) 145 potassium aspartate, 2 MgCl2, 10 HEPEs, 10 K2EGTA, and 5.5 CaCl2 (1 μM free Ca2+), pH 7.2, 290–310 mOsm. IKv,m currents were recorded with a fluoride-based internal solution. To reduce currents from native chloride channels in COS-7, sodium aspartate Ringer was used as an external solution (in mM) 160 sodium aspartate, 4.5 KC1, 2 CaCl2, 1 MgCl2, and 5 HEPEs, pH 7.4, 290–310 mOsm. For Jurkat T cells, potassium aspartate Ringer was used as an external solution with K+ instead of Na+ (164.5 mOsm). IKv,m currents were elicited by 200-ms voltage ramps from −120 to 40 mV applied every 10 s, and the reduction of slope conductance at −80 mV by the toxin was taken as a measure of channel block. BK,Ca currents were elicited by 200-ms voltage ramps from −80 to 80 mV applied every 30 s, and channel block measured at 35 mV. The inward rectifier (rKir 2.1) in RBL cells was studied in sodium aspartate Ringer with a potassium aspartate-based pipette solution containing 50 nM free Ca2+.

**Selective in Vivo Blockade of SKCa2 Channels by Lei-Dab7 in Mice**—Lei-Dab7 was administered to 25-g C57/B6 mice via the intracerebroventricular route, and the LD50 was determined (33). Groups of four mice per dose were injected with 5 μl of the peptide solution containing 0.1% (w/v) bovine serum albumin and 0.9% (w/v) sodium chloride.

**RESULTS**

**Lei and PO5 Are Potent Inhibitors of Human SKCa,m Channels**—Six peptide toxins from scorpion venom have been identified as SKCa,m channel blockers based on 125I-apamin displacement experiments on rat brain synaptosomes (34). Fig. 1 shows the sequence alignment of these toxins (35), three of which (Lei, PO5, and Pi1) contain a motif (RXCQ) reported to be important for binding to SKCa channels. (20, 36). Apamin contains an RRCQ sequence (Fig. 1), which has a spatial arrangement similar to that of the RMCQ motif in Lei (Fig. 2). Lei is reported to block the well characterized human SKCa2 channel in Jurkat T cells with picomolar potency (15, 22, 23). As the first step in our strategy to design a specific SKCa2 inhibitor, we compared the potency of Lei with that of PO5, a toxin that differs from Lei only at positions 1, 2, 7, and 24 (Fig. 3). The differing residues of Lei and PO5 are boxed. The conserved RXCQ motif is boxed. The differing residues of Lei and PO5 are highlighted. MTX, maurotoxin.

**FIG. 1. Sequence alignment of known SKCa blockers showing subfamilies 4, 5, 6, and 8 from scorpion venom (28) and apamin. The conserved RXCQ motif is boxed. The differing residues of Lei and PO5 are highlighted. MTX, maurotoxin.**

**FIG. 2. Superimposition of the backbone structures of Lei (yellow) and apamin (blue). The RXCQ motif is boxed. Inset, RXCQ motif in both toxins on a larger scale.**

**Scorpion toxins**

**Subfamily 5**

| Lei-I | APFRLEMCQGSCRSLLGLGGKCIKDRCRVKH |
| PO5  | TVCTNLEMCQGSCRSLLGLGGKCIKDRCRVKH |

**Subfamily 4**

| Ts-Kappa | TVVIGQCRYBSPCYSACQKLLGATGCTNRCDC |

**Subfamily 6**

| Pi1    | PLVKCRGTSCEGCPPQCGQGCQPNKSNCKMCYGC |
| MTX    | VSCTGSKDCYAPCQRTGCPNNAKCNKSCCKYGC |

**Subfamily 8**

| PO1    | VECKDCPEBHCTQEGKQACCKNLDKCVCEPI |
were next evaluated on cloned SKCa1 and SKCa3 expressed in COS-7 cells. SKCa1 (Fig. 3A, middle) and SKCa3 (right) K⁺ currents elicited with 1 mM Ca²⁺ in the pipette solution reversed at −80 mV in the presence of external sodium Ringer. Lei and PO5 blocked SKCa3 but were ineffective on SKCa1 (Fig. 3A and Table I). Comparison of the dose-response curves of SKCa3 showed Lei to be 25-fold more effective than PO5 (Fig. 3B and Table I). For reasons that remain unclear, four other scorpion toxins (maurotoxin, Pi1, PO1, and Ts) reported to be highly active in ¹²⁵I-apamin displacement assays (20, 34, 36) had little or no blocking activity on SKCa2 or SKCa3 (Table I). Thus, the two most potent scorpion peptides, Lei and PO5, exhibited significantly different blocking potencies on SKCa2 and SKCa3 despite differing at only four positions. 

![SKCa2 and Kv1.3](image1)

**Lei-Dab⁷**, a Selective Blocker of SKCa2

**TABLE I**

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Source</th>
<th>¹²⁵I-displacement</th>
<th>SKCa2 (Jurkat)</th>
<th>SKCa3 (COS-7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leiurotoxin I (scyllatoxin)</td>
<td><em>Leirus quinquenstriatius hebraeus</em></td>
<td>N/A</td>
<td>0.2 ± 0.05 nM</td>
<td>1.1 ± 0.2 nM</td>
</tr>
<tr>
<td>PO5</td>
<td><em>Androctonus mauretanicus mauretanicus</em></td>
<td>20 pm</td>
<td>22 ± 0.5 nM</td>
<td>25 ± 2.5 nM</td>
</tr>
<tr>
<td>Tsc</td>
<td><em>Tityus serrutatus</em></td>
<td>300 pm</td>
<td>80 ± 11 nM</td>
<td>197 ± 8 nM</td>
</tr>
<tr>
<td>P1-OH</td>
<td><em>Pandinus imperator</em></td>
<td>N/A</td>
<td>&gt;1 μM</td>
<td>330 nM</td>
</tr>
<tr>
<td>P1-NH₂</td>
<td><em>Pandinus imperator</em></td>
<td>0.55 pm</td>
<td>100 ± 30 nM</td>
<td>250 nM</td>
</tr>
<tr>
<td>Maurotoxin (MTX)</td>
<td><em>Scorpio maurus palmatus</em></td>
<td>5–12 nM</td>
<td>1 μM</td>
<td>&gt;1 μM</td>
</tr>
<tr>
<td>PO1</td>
<td><em>Androctonus mauretanicus mauretanicus</em></td>
<td>100 nM</td>
<td>&gt;1 μM</td>
<td>&gt;1 μM</td>
</tr>
</tbody>
</table>

For further details, please refer to the figure and text provided. The figure shows typical current traces on the three SKCa channels showing block with Lei and PO5. The table compares the competition data on rat brain synaptosomes of indicated toxins with KD values by patch clamp. The figure illustrates the dose-response curves of Lei and PO5 on SKCa1–3. The table includes a comparison of the dose-response curves of Lei and PO5 on SKCa1–3. The figure and table provide a comprehensive overview of the effects of Lei and PO5 on SKCa1–3 channels.
alterations in the side chains at these positions may allow PO5 to fit more tightly within the channel-binding pocket due to either shorter side-chain size or local change in backbone conformation (37, 38). Similar results were obtained with these PO5 mutants on SKCa3 (Fig. 4B). The reverse mutation in Lei (Lei-M7R) reduced potency on both SKCa2 and SKCa3 (data not shown). These results indicate that Ala1, Phe2, and Met7 underlie the increased affinity of Lei over PO5 for SKCa2 and SKCa3. These three residues form a localized binding pocket that may represent an important contact point with SKCa channels (Fig. 5, A and B).

**Lei-Dab7 Is a Highly Selective Inhibitor of SKCa2**—Positions 6 and 7 of Lei are part of the conserved RXCQ motif (Fig. 1). To define the role of the residues in this motif, a series of Lei mutants was made at positions 6 and 7 to probe the toxin-channel interaction. Charge-neutralization mutations at position 6 that retained size (Arg6 → leucine or citrulline) reduced toxin potency 70–180-fold for both channels (Fig. 6), indicating the need for a charged residue at this position. Substitution of the unbranched lysine at position 6, a mutation that retained charge but decreased size, also reduced toxin affinity for both channels (20–35-fold), whereas the introduction of the positively charged, bulky branched unnatural amino acid, homoarginine, caused a 1000-fold decrease in toxin potency (Fig. 6). Thus, substitutions at position 6 are not tolerated, and the arginine has the optimum size, charge, and branching required for interaction with SKCa channels. Furthermore, when residues at positions 6 and 7 were exchanged, the Lei-R6M+M7R double mutant was considerably less potent than the native toxin on SKCa2 and SKCa3 (Kd = 9.5 and 65 nM, respectively), establishing the importance of the relative locations of Arg6 and Met7 in the RXCQ motif.

We next turned our attention to position 7 in the RXCQ motif and found our first promising lead. Introduction of positively charged lysine at position 7 (Lei-M7K) yielded a mutant that blocked SKCa2 35-fold more potently than SKCa3 (Fig. 7). In an attempt to further enhance this difference, we generated two additional Lei mutants in which Met7 was replaced by smaller positively charged unnatural amino acids diaminopropionate (Lei-Dapa7) and diaminobutanoate (Lei-Dab7). Lei-Dapa7 blocked Jurkat SKCa2 ∼350-fold more potently than SKCa3, whereas Lei-Dab7 was ∼650-fold more effective. Similar results...
were obtained with the cloned SKCa2 channel expressed in COS-7 cells (Table II). Lei-Dab7 was also ineffective against hSKCa1, hIKCa1, hSlo, Kv, and Kir channels, establishing its specificity for SKCa2 (Table II).

Asn\textsuperscript{367} in the SKCa2 Pore Region Is Important for Lei-Dab\textsuperscript{7} Selectivity—Because Lei-Dab\textsuperscript{7} and apamin share the critical RXCQ channel-binding motif (Figs. 1 and 2), it is likely that Lei-Dab\textsuperscript{7}, like apamin (39), binds to residues in the external S5-Pore-S6 region of the channel. Human SKCa2 and SKCa3 differ at only two positions in the pore region (Fig. 8A). To determine whether one or both these residues contribute to Lei-Dab\textsuperscript{7} selectivity (Fig. 8, B and C), we replaced these two residues in SKCa3 (Val\textsuperscript{485} and His\textsuperscript{521}) with the corresponding residues of SKCa2 (Ala\textsuperscript{331} and Asn\textsuperscript{367}), individually or together. Lei-Dab\textsuperscript{7} blocked SKCa3-H521N (\(K_d = 20 \pm 4.7 \text{nM}\)) and SKCa3-V485A + H521N (\(K_d = 7.5 \pm 1.2 \text{nM}\)) with nearly the same potency as SKCa2 (Fig. 8, D and E, and Table II), whereas SKCa3-V485A did not produce functional channels. In mutant cycle studies with the SKCa3-H521N and SKCa3-V485A+H521N mutants, residue 7 of Lei-Dab\textsuperscript{7} was found to couple tightly with His\textsuperscript{521} (\(\Delta \Delta G = 2.4\)), suggesting that these two residues lie in close proximity to each other. We conclude that Lei-Dab\textsuperscript{7} binds to the external vestibule of SKCa channels and that Asn\textsuperscript{367} (the residue corresponding to His\textsuperscript{521} of hSKCa3) in SKCa2 contributes to Lei-Dab\textsuperscript{7} selectivity.

Selective in Vivo Blockade of Homotetrameric SKCa2 Channels by Lei-Dab\textsuperscript{7} Does Not Cause Gross Neurotoxicity—To evaluate the central nervous system effects of specific in vivo blockade of SKCa2 homotetramers, Lei-Dab\textsuperscript{7} was administered via the intracerebroventricular route to mice. At a concentration (10 ng (or 300 nM assuming a brain liquid volume of 10 \(\mu\)l)) that would selectively block 99\% of SKCa2 homotetramers, no gross central nervous system toxicity was observed.

<table>
<thead>
<tr>
<th>Side chain at position 7</th>
<th>SKCa2</th>
<th>SKCa3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native toxin-Methionine</td>
<td>(CH\textsubscript{3})\textsuperscript{5}</td>
<td>0.2 ± 0.05 nM (n = 12)</td>
</tr>
<tr>
<td>Leucine</td>
<td>(CH\textsuperscript{3})\textsuperscript{5}</td>
<td>0.55 ± 0.05 nM (n = 5)</td>
</tr>
<tr>
<td>Lysine</td>
<td>(CH\textsuperscript{3})\textsuperscript{5}</td>
<td>3 ± 0.5 nM (n = 5)</td>
</tr>
<tr>
<td>Dap</td>
<td>(CH\textsuperscript{3})\textsuperscript{5}</td>
<td>5.5 ± 0.8 nM (n = 5)</td>
</tr>
<tr>
<td>Dab</td>
<td>(CH\textsuperscript{3})\textsuperscript{5}</td>
<td>3.8 ± 1.1 nM (n = 11)</td>
</tr>
</tbody>
</table>

Fig. 7. Effect of substitutions at position 7 of Lei in blocking SKCa2 (left) and SKCa3 (right). The respective \(K_d\) values is shown on the right. Numbers in parentheses indicate the number of times tested.

Fig. 8. Effect of replacement of differing residues in the S5-Pore-S6 region in SKCa3 with the corresponding residues in SKCa2. A, sequence alignment of SKCa2 and SKCa3 S5-Pore-S6 region with the two differing residues underlined. B, 10 nm Lei-Dab\textsuperscript{7} on native SKCa2 (\(K_d = 3.8 \pm 0.5 \text{nM}\)) and 1000 nm Lei-Dab\textsuperscript{7} on hSKCa3 (\(K_d = 2500 \pm 500 \text{nM}\)). D, 100 nm Lei-Dab\textsuperscript{7} on hSKCa3-H521N (\(K_d = 20 \pm 4.7 \text{nM}\)). E, 10 nm Lei-Dab\textsuperscript{7} on hSKCa3-V485A+H521N (\(K_d = 7.5 \pm 1.2 \text{nM}\)).
observed. At a higher concentration (50 ng (or 1500 nM)), all six animals became hyperexcitable, developed convulsions followed by paralysis lasting 15 h, and then fully recovered. At much higher concentrations (80 ng (or 2400 nM)), 50% of the animals died within 2 h, and 100% lethality was observed in 1 h at 100 ng (3 μM). The neurotoxicity observed at Lei-Dab7 concentrations higher than 1500 nM may be due to the blockade of SKCa1 and/or SKCa3 homotrameric channels (Table II), although we cannot exclude the contribution of heteromultimeric SKCa channels containing SKCa2 subunits.

REFERENCES

43. Dauplais, M., Lecoq, A., Song, J., Cotton, J., Jamin, N., Guilpin, B., Roumestand, C., Vita, C., de Medeiros, C. L. C., Rowan, E. G., Harvey, A. L.,
Lei-Dab\textsuperscript{7}, a Selective Blocker of SKCa\textsubscript{2}

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