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 lack of specific inhibitors. Here we describe the CQ motif, potently blocked human SKCa2 and SKCa3 but not SKCa1, whereas maurotoxin, Pi1, Tsx, and PO5 were ineffective. Lei blocked these channels more potently than PO5 because of the presence of Ala1′, Phe5′, 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 (Kd = 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.
Lei-Dab7, a Selective Blocker of SKCa2

EXPERIMENTAL PROCEDURES

Peptide Synthesis—N-α-Fmoc-α-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 (Pepgin, 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-pentamethylchromane-6-sulfonil for Arg and homoarginine; tert-butyloxycarbonyl for ornithine, Lys, and homolysine, and 1-(4-diethylamino-2,6-dioxycyclohex-1-yliden)-3-methylbutyl for Dab and diaminopropionic acid (Dapa). The reduced reagents were obtained from Sigma.

Biochemicals) with the respective DNA in serum-free OptiMEM medium (Life Technologies, Inc.) as per the manufacturer instructions. GFP-positive cells were used for electrophysiological studies at 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 Aquaapor ODS 20 μm, 250 × 10 mm). The homogeneity (>99%) and identity of the peptides were verified by: (i) analysis of C18 reversed-phase HPLC, (ii) amino acid content determination after acidolysis, and (iii) 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 at densities of 1–9 × 10⁶ in a 37 °C humidified incubator with 5% CO₂. COS-7 and PC12 cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 2 mM glutamine and split twice weekly. Unless otherwise specified, all reagents were obtained from Sigma.

Clones and Mutants—The cloning of human SKCa3 containing 19 polyglutamines in the N terminus (GenBankTM AF031815, AJ251016) and IKCa1 (GenBankTM AF030201) has been reported previously (30–32). Selection of subfamilies of SKCa1 was amplified from a 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 pEFGFP-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 FuGene6 (Roche Molecular Biochemicals) with the respective DNA in serum-free OptiMEM medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 10 mM HEPES (PerkinElmer Life Sciences, Inc., Shelton, CT, Novabiochem, Fitchburg, WI, and Biozym, Bad Homburg, Germany). The side-chain protecting groups used for trifunctional residues were: 2,2,5,7,8-pentamethylchromane-6-sulfonil for Arg and homoarginine; tert-butyloxycarbonyl for ornithine, Lys, and homolysine, and 1-(4-diethylamino-2,6-dioxycyclohex-1-yliden)-3-methylbutyl for Dab and diaminopropionic acid (Dapa). The reduced peptides were dissolved at 1 mM in 0.2% Tris-HCl buffer, pH 8.3, and stirred under air to allow folding/oxidation (48 h, 25 °C). The folds/oxidized toxins and structural analogs were purified to homogeneity by reversed-phase high-pressure liquid chromatography (HPLC) (Pepgin Life Sciences), C18 Aquaapor ODS 20 μm, 250 × 10 mm). The homogeneity (>99%) and identity of the toxins were verified by: (i) analysis of C18 reversed-phase HPLC, (ii) amino acid content determination after acidolysis, and (iii) mass analysis by matrix-assisted laser desorption ionization-time of flight mass spectrometry.

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Electrophysiology—Cells were studied in the whole cell configuration using the patch clamp technique. The patch clamp potential was in all configurations was −80 mV. For measurement of IKCa, SKCa, and BKCa currents, we used an internal pipette solution containing (in mM) 145 potassium aspartate, 2 MgCl₂, and 5 HEPES, pH 7.2, 290–310 mM. BKCa currents were recorded with 200-mV 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. BKCa 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 (rKir2.1) in RBL cells was studied in sodium aspartate Ringer with a potassium aspartate-based pipette solution containing 50 mM free Ca²⁺.

**RESULTS**

Selective in Vivo Blockade of SKCa2 Channels by Lei-Dab7 in Mice—Lei-Dab7 was administered to 25-g C57/BL6 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.

Lei and PO5 Are Potent Inhibitors of Human SKCa Channel—Six peptide toxins from scorpion venom have been identified as SKCa channel blockers based on [28]–aminopaf displacement studies 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 (RCXQ) reported to be important for binding to SKCa channels. (36). Apamin contains an RRCQ sequence (Fig. 1), which has a spatial arrangement similar to that of the RMQ 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 toxins Lei and PO5 are equipotent (28-fold) at blocking the well characterized human SKCa2 current. Following break-in with 1 mM Ca²⁺ in the pipette solution, SKCa2 currents were seen at negative potentials at potential more negative than −40 mV. K⁺ currents were mainly due to Kv1.3 with minimal contribution from K1.8 (Fig. 3A, left). The SKCa2 component was blocked by Lei and PO5, whereas the Kv1.3 current was unaffected (Fig. 3A, left). Dose-response curves showed that Lei blocked Jurkat SKCa2 with Kᵥ values consistent with published data on the cloned and native channel (15, 22, 23) and with ~100-fold greater potency than PO5 (Fig. 3B and Table I). Lei and PO5
were next evaluated on cloned SKCa1 and SKCa3 expressed in COS-7 cells. SKCa1 (Fig. 3A, middle) and SKCa3 (Fig. 3A, 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). Typical SKCa1–3 currents were studied in the whole cell configuration of the patch clamp technique. Recordings were done with 1 μM free calcium as the internal pipette solution, and currents were elicited by voltage ramps from −120 to 40 mV. SKCa1 and SKCa3 were expressed transiently in COS-7 cells. An external solution containing sodium aspartate (5 mM potassium aspartate) was used for the recordings, and the degree of block was measured as the decrease in slope conductance at −80 mV. Jurkat T-cells were used to assess the effect on endogenous hSKCa2 with symmetric internal and external potassium aspartate (165 mM potassium aspartate).

TABLE I
Comparison of competition data on rat brain synaptosomes of indicated toxins (20, 34, 36) with \( K_d \) values by patch clamp on hSKCa2 and hSKCa3

<table>
<thead>
<tr>
<th>Toxin</th>
<th>Source</th>
<th>( 1^{25}I )-displacement</th>
<th>( K_d ) hSKCa2 (Jurkat)</th>
<th>( K_d ) hSKCa3 (COS-7)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leiurotoxin I (scyllatoxin)</td>
<td>Leiurus quinquestriatus hebraeus</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>Androctonus mauretanicus mauretanicus</td>
<td>20 pm</td>
<td>22 ± 0.5 nM</td>
<td>25 ± 2.5 nM</td>
</tr>
<tr>
<td>Tsc</td>
<td>Titys serrulatus</td>
<td>300 pm</td>
<td>80 ± 11 nM</td>
<td>197 ± 8 nM</td>
</tr>
<tr>
<td>P1-OH</td>
<td>Pandinus imperator</td>
<td>N/A</td>
<td>&gt;1 μM</td>
<td>330 nM</td>
</tr>
<tr>
<td>P1-NH₂</td>
<td>Pandinus imperator</td>
<td>0.55 μM</td>
<td>100 ± 30 nM</td>
<td>250 nM</td>
</tr>
<tr>
<td>Maurotoxin (MTX)</td>
<td>Scorpio maurus palmaurus</td>
<td>5–12 nm</td>
<td>1 μM</td>
<td>&gt;1 μM</td>
</tr>
<tr>
<td>PO1</td>
<td>Androctonus mauretanicus mauretanicus</td>
<td>100 nm</td>
<td>&gt;1 μM</td>
<td>&gt;1 μM</td>
</tr>
</tbody>
</table>

Lei-Dab⁷, a Selective Blocker of SKCa2

were next evaluated on cloned SKCa1 and SKCa3 expressed in COS-7 cells. SKCa1 (Fig. 3A, middle) and SKCa3 (Fig. 3A, right) K⁺ currents elicited with 1 μM 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, P1, PO1, and Tsc) reported to be highly active in \( 1^{25}I \)-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.

\[ \text{Ala}^1, \text{Phe}^2, \text{and Met}^7 \text{ in Lei Are Responsible for the Enhanced Potency of Lei over PO5} \]—To define the residues responsible for the increased affinity of Lei for SKCa channels, we replaced each of the four differing residues in PO5 with the corresponding residue in Lei. PO5-V24D blocked SKCa2 and SKCa3 with a potency comparable with native PO5 (Fig. 4A). PO5-T1A, PO5-V2F, and PO5-R7M blocked SKCa2 with potencies approaching that of Lei. The improved potency of all three PO5 mutants to nearly that of Lei suggests that any of the indicated
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 SKCa2 (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, a Selective Blocker of SKCa2**

Positions 6 and 7 of Lei are part of the conserved R\(\times\)CQ 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 (Arg\(^6\) \rightarrow 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 (\(K_d = 9.5\) and 65 nM, respectively), establishing the importance of the relative locations of Arg\(^6\) and Met\(^7\) 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-Dapa\(^7\)) and diaminobutanoate (Lei-Dab\(^7\)). Lei-Dapa\(^7\) blocked Jurkat SKCa2 \(\sim\)350-fold more potently than SKCa3, whereas Lei-Dab\(^7\) was \(\sim\)650-fold more effective. Similar results

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**Fig. 4.** Histograms showing the effect of replacement of Thr\(^1\), Val\(^2\), Arg\(^7\), and Val\(^24\) in PO5 with the corresponding residues in Lei in blocking SKCa2 (A) and SKCa3 (B). The \(K_d\) values of native toxin is shown for comparison.

**Fig. 5.** Space-filling models of Lei (left) and PO5 (right) showing residues at positions 1, 2, 7, and 24 (A) and the conserved R\(\times\)CQ motif (B). \(X =\) methionine in Lei and arginine in PO5.

**Fig. 6.** Effect of substitutions at position 6 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.
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).

Asn367 in the SKCa2 Pore Region Is Important for Lei-Dab7 Selectivity—Because Lei-Dab7 and apamin share the critical RXCQ channel-binding motif (Figs. 1 and 2), it is likely that Lei-Dab7, 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-Dab7 selectivity (Fig. 8, B and C), we replaced these two residues in SKCa3 (Val485 and His521) with the corresponding residues of SKCa2 (Ala331 and Asn367), individually or together. Lei-Dab7 blocked SKCa3-H521N (Kd = 20 ± 4.7 nM) and SKCa3-V485A + H521N (Kd = 7.5 ± 1.2 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-Dab7 was found to couple tightly with His521 (ΔΔG = 2.4), suggesting that these two residues lie in close proximity to each other. We conclude that Lei-Dab7 binds to the external vestibule of SKCa channels and that Asn367 (the residue corresponding to His521 of hSKCa3) in SKCa2 contributes to Lei-Dab7 selectivity.

Selective in Vivo Blockade of Homotetrameric SKCa2 Channels by Lei-Dab7 Does Not Cause Gross Neurotoxicity—To evaluate the central nervous system effects of specific in vivo blockade of SKCa2 homotetramers, Lei-Dab7 was administered via the intracerebroventricular route to mice. At a concentration (10 ng (or 300 nM assuming a brain liquid volume of 10 μl)) that would selectively block 99% of SKCa2 homotetramers, no gross central nervous system toxicity was observed.

**TABLE II**

Selectivity of Lei-Dab7 on a panel of channels Kd indicated on the right

<table>
<thead>
<tr>
<th>Channel</th>
<th>Lei-Dab7 Kd</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>KCa channels</strong></td>
<td></td>
</tr>
<tr>
<td>hSKCa1</td>
<td>6000 ± 800 nM</td>
</tr>
<tr>
<td>hSKCa2 (Jurkat)</td>
<td>3.8 ± 0.5 nM</td>
</tr>
<tr>
<td>hSKCa2 (cloned)</td>
<td>5.5 ± 0.7 nM</td>
</tr>
<tr>
<td>hSKCa3</td>
<td>2500 ± 500 nM</td>
</tr>
<tr>
<td>hIKCa1</td>
<td>No effect at 1 μM</td>
</tr>
<tr>
<td>hSlo</td>
<td>No effect at 1 μM</td>
</tr>
<tr>
<td><strong>Kv channels</strong></td>
<td></td>
</tr>
<tr>
<td>mKv1.1</td>
<td>No effect at 1 μM</td>
</tr>
<tr>
<td>mKv1.3 (cloned)</td>
<td>No effect at 1 μM</td>
</tr>
<tr>
<td>hKv1.3 (Jurkat)</td>
<td>No effect at 1 μM</td>
</tr>
<tr>
<td>hKv1.5</td>
<td>No effect at 1 μM</td>
</tr>
<tr>
<td>rKv1.2 and Kv2.1 (PC12)</td>
<td>No effect at 1 μM</td>
</tr>
<tr>
<td><strong>Inward rectifier</strong></td>
<td></td>
</tr>
<tr>
<td>rKir2.1 (RBL cells)</td>
<td>No effect at 1 μM</td>
</tr>
</tbody>
</table>
Therefore, the architecture of the toxin-binding surface in the aromatic residue with a neighboring invariant lysine (43).

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., Lecooq, A., Song, J., Cotton, J., Jamin, N., Gilquin, B., Roumestand, C., Vita, C., de Medeiros, C. L. C., Rowan, E. G., Harvey, A. L., Acknowledgments—We thank Chialing Wu and Dr. Luette Forrest for technical assistance, Dr. Pascal Mansuelle for amino acid analysis and Edman sequencing, and Prof. Herve Rochat for constant support.
Lei-Dab$^7$, a Selective Blocker of SKCa2


