K⁺ channel types targeted by synthetic OSK1, a toxin from *Orthochirus scrobiculosus* scorpion venom

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OSK1 (α -KTx3.7) is a 38-residue toxin cross-linked by three disulphide bridges that was initially isolated from the venom of the Asian scorpion *Orthochirus scrobiculosus*. OSK1 and several structural analogues were produced by solid-phase chemical synthesis, and were tested for lethality in mice and for their efficacy in blocking a series of 14 voltage-gated and Ca²⁺-activated K⁺ channels *in vitro*. In the present paper, we report that OSK1 is lethal in mice by intracerebroventricular injection, with a LD₅₀ (50 % lethal dose) value of 2 μ g/kg. OSK1 blocks K_v1.1, K_v1.2, K_v1.3 channels potently and K_{Ca}3.1 channel moderately, with IC₅₀ values of 0.6, 5.4, 0.014 and 225 nM respectively. Structural analogues of OSK1, in which we mutated positions

16 (Glu¹⁶ → Lys) and/or 20 (Lys²⁰ → Asp) to amino acid residues that are conserved in all other members of the α -KTx3 toxin family except OSK1, were also produced and tested. Among the OSK1 analogues, [K₁₆,D₂₀]-OSK1 (OSK1 with Glu¹⁶ → Lys and Lys²⁰ → Asp mutations) shows an increased potency on K_v1.3 channel, with an IC₅₀ value of 0.003 nM, without loss of activity on K_{Ca}3.1 channel. These data suggest that OSK1 or [K₁₆,D₂₀]-OSK1 could serve as leads for the design and production of new immunosuppressive drugs.

Key words: Ca²⁺-activated K⁺ channel, OSK1, peptide synthesis, scorpion toxin, voltage-gated K⁺ channel.

INTRODUCTION

OSK1 is a toxin initially purified from the venom of the central Asian scorpion *Orthochirus scrobiculosus* [1]. It is a 38-residue peptide cross-linked by three disulphide bridges that are organized according to the conventional pattern of the three-disulphide-bridged scorpion toxins (i.e. C1–C4, C2–C5 and C3–C6) [1,2]. The 3D (three-dimensional) structure of natural OSK1 in solution, determined by ¹H-NMR [1], shows that the toxin adopts the α/β scaffold (i.e. an α -helix connected to an antiparallel β -sheet by two disulphide bridges) common to most characterized scorpion toxins, independent of their size and selectivity towards the various ion channels [3]. Together with KTx1 (kaliotoxin 1) [4], AgTx1–3 (agitoxin 1–3) [5] and BmKTx (*Buthus martensi* kaliotoxin) [6], OSK1 belongs to a structural class referred to as α -KTx3 toxins that share between 68 and 90 % sequence identity with each other [7]. According to this nomenclature, OSK1 has been termed α -KTx3.7 [8]. OSK1 has been described previously to block apamin-insensitive small-conductance Ca²⁺-activated channels in neuroblastoma-glioma NG108-15 hybrid cells [9], which are most likely to be identical with K_{Ca}3.1 channel. Although the solution structure of OSK1 has been solved [1] (Protein Data Bank code 1SCO), characterization of its pharmacological properties still remained poor. In the present paper, we describe the chemical production and characterization of OSK1. The synthetic product was verified to be indistinguishable from natural OSK1 using two-dimensional ¹H-NMR, and an extensive analysis of its pharmacological profile

was achieved, demonstrating that it behaves *in vitro* as a potent inhibitor of a large array of K⁺ channel subtypes. Structural analogues of OSK1, in which we introduced selective mutations of amino acid residues that are conserved in all members of the α -KTx3 toxin family, except OSK1, were also produced and tested for their pharmacology on various K⁺ channel types.

EXPERIMENTAL

Materials

Fmoc (N^α-fluoren-9-ylmethoxycarbonyl)-L-amino acids and reagents used for peptide synthesis were obtained from PerkinElmer (Shelton, CT, U.S.A.). Fmoc-Lys(*t*-butyloxycarbonyl)-2-chlorotrityl resin was purchased from Advanced ChemTech (Cambridge, U.K.). Solvents were analytical grade products from SDS (Peypin, France).

Chemical synthesis and characterization of OSK1 and related analogues

OSK1, [K₁₆]-OSK1, [D₂₀]-OSK1, [K₁₆,D₂₀]-OSK1, [P₁₂,K₁₆,D₂₀]-OSK1 and [K₁₆,D₂₀,Y₃₆]-OSK1 (where P₁₂ is an Arg¹² → Pro mutation, K₁₆ is a Glu¹⁶ → Lys mutation, D₂₀ is a Lys²⁰ → Asp mutation and Y₃₆ is a Thr³⁶ → Tyr mutation) were all produced by chemical solid-phase synthesis using an automated peptide synthesizer (Model 433A, Applied Biosystems Inc.). In all cases, peptide chains were assembled stepwise on 0.40 mmol of

Abbreviations used: AgTx, agitoxin; [Ca²⁺]_i, internal Ca²⁺ concentration; FCS, foetal calf serum; Fmoc, N^α-fluoren-9-ylmethoxycarbonyl; GFP, green fluorescent protein; K_{Ca}2.1, K_{Ca}2.2 and K_{Ca}2.3 channels, type 1, 2 and 3 small-conductance Ca²⁺-activated K⁺ channels (SK1, SK2 and SK3 respectively); K_{Ca}3.1 channel, type 1 intermediate-conductance Ca²⁺-activated K⁺ channel (IK1); K_{Ca}1.1 channel, large-conductance Ca²⁺-activated K⁺ channel (BK or Slo α); K_v channel, mammalian voltage-gated K⁺ channel; K_v11.x channel, human *ether-a-go-go*-related K⁺ channel (HERG); KTx1, kaliotoxin 1; LD₅₀, 50 % lethal dose; MALDI-TOF, matrix-assisted laser-desorption ionization-time-of-flight; NOE, nuclear Overhauser effect; OSK1, toxin 1 from the scorpion *Orthochirus scrobiculosus* (α -KTx3.7); *t*-Bu, *t*-butyl; TFA, trifluoroacetic acid; 3D, three-dimensional.

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Fmoc-Lys(*t*-butoxycarbonyl)-2-chlorotrityl resin (1% cross-linked; 0.69 mmol of amino group/g) using 1 mmol of Fmoc-L-amino acid derivatives [10]. The side-chain-protecting groups used for trifunctional residues were: Trt (trityl) for cysteine, asparagine, histidine and glutamine; *t*-Bu (*t*-butyl) for serine, threonine, tyrosine, aspartate and glutamate; Pmc (pentamethylchroman) for arginine; and Boc (*t*-butoxycarbonyl) for lysine. N^{α} -amino groups were deprotected by successively treating with 18 and 20% (v/v) piperidine/*N*-methylpyrrolidone for 3 and 8 min respectively. After three washes with *N*-methylpyrrolidone, the Fmoc-amino acid derivatives were coupled (20 min) as their hydroxybenzotriazole-active esters in *N*-methylpyrrolidone (2.5-fold excess). After complete assembly of the peptides, and removal of their N-terminal Fmoc groups, the peptide resins (approx. 2.4 g) were treated under stirring for 2.5 h at 25°C with mixtures of TFA (trifluoroacetic acid)/water/thioanisole/ethanedithiol (88:5:5:2, by vol.) in the presence of crystalline phenol (2.25 g) in final volumes of 30 ml/g of peptide resins. The peptide mixtures were filtered, precipitated and washed twice with cold diethyl oxide. The crude peptides were pelleted by centrifugation (3000 g for 10 min). The crude peptides were then dissolved in water and freeze-dried. Reduced peptides were solubilized (approx. 0.8 mM) in 0.2 M Tris/HCl buffer, pH 8.3, for oxidative folding (40–140 h, depending on the analogue, 22°C). All peptides were purified to homogeneity by reversed-phase HPLC (PerkinElmer; C₁₈ Aquapore ODS 20 μ m, 250 mm $\times$ 10 mm) by means of a 60-min linear gradient of 0.08% (v/v) TFA/0–35% acetonitrile in 0.1% (v/v) TFA/water at a flow rate of 6 ml/min (λ = 230 nm). The purity and identity of the peptides were assessed by: (i) analytical C₁₈ reversed-phase HPLC (Merck; C₁₈ Lichrospher 5 μ m, 4 mm $\times$ 200 mm) using a 60-min linear gradient of 0.08% (v/v) TFA/0–60% acetonitrile in 0.1% (v/v) TFA/water at a flow rate of 1 ml/min; (ii) Edman sequencing; and (iii) molecular mass determination by MALDI-TOF (matrix-assisted laser-desorption ionization–time-of-flight) MS.

Conformational analyses of OSK1 by two-dimensional ¹H-NMR and of its analogues by one-dimensional ¹H-NMR

Peptides were dissolved in a mixture of H₂O/²H₂O (9:1, v/v) at final concentrations of 10^{−3} M (OSK1) or 50 μ M (OSK1 analogues). All ¹H-NMR measurements were obtained on a Bruker DRX 500 spectrometer equipped with an HCN probe, and self-shielded triple-axis gradients were used. The experiments were performed at 300 K.

Neurotoxicity of the peptides in mice

OSK1 and its analogues were tested *in vivo* for toxicity by determining the LD₅₀ (50% lethal dose) after intracerebroventricular injection into 20 g C57/BL6 mice (approved by the French ethics committee; animal testing agreement number 006573 delivered by the National Department 'Santé et Protection Animales, Ministère de l'Agriculture et de la Pêche'). Groups of four to six mice per dose were injected with 5 μ l of peptide solution containing 0.1% (w/v) BSA and 0.9% (w/v) NaCl.

Cells

L929 and MEL (murine erythroleukaemia) cells stably expressing mouse K_v1.3 (mK_v1.3), human K_v1.5 (hK_v1.5) and mouse K_v3.1 (mK_v3.1) channels, and COS-7 cells were maintained in DMEM (Dulbecco's modified Eagle's medium) with Earle's salts (Gibco, Paisley, U.K.) and 10% (v/v) heat-inactivated FCS (foetal calf serum) (PAA Laboratories, Pasching, Austria) as described previously [11]. The tsA cell line expressing human K_{Ca}3.1

(hK_{Ca}3.1) channels was a gift from Dr Daniel Devor (University of Pittsburgh, Pittsburgh, PA, U.S.A.) and was maintained in MEM (minimal essential medium) with Earle's salts supplemented with Glutamax-I (Gibco), 10% heat-inactivated FCS and 200 μ g/ml geneticin (G418). Cells were kept at 37°C in a humidified incubator containing 5% (for the L929 cell line expressing mK_v3.1 channel) or 10% CO₂ (for the other cell lines).

LTK− cells (lacking leucocyte tyrosine kinase) expressing human K_v1.4 (hK_v1.4) channel were obtained from Professor Michael Tamkun (Colorado State University, Fort Collins, CO, U.S.A.), CHL (Chinese-hamster lung) cells expressing mouse K_v1.7 (mK_v1.7) channel were from Vertex Pharmaceuticals (San Diego, CA, U.S.A.), HEK-293 (human embryonic kidney) cells expressing human K_{Ca}1.1 (hBK or hSlo α) channel were from Dr Andrew Tinker (Centre for Clinical Pharmacology, University College London, London, U.K.), HEK-293 cells expressing K_v11.x [HERG (human *ether-a-go-go*-related gene)] channel were from Professor Craig T. January (Department of Medicine, University of Wisconsin, Madison, WI, U.S.A.), and HEK-293 cells expressing human K_{Ca}2.1 (hK_{Ca}2.1) and rat K_{Ca}2.2 (rK_{Ca}2.2) channels were from Dr Khaled Houamed (University of Chicago, Chicago, IL, U.S.A.).

Transfection

The GFP (green fluorescent protein)-IRES vector (Invitrogen, Heidelberg, Germany) containing the coding sequence of mouse K_v1.1 (mK_v1.1) channel was transfected into COS-7 cells, while the pcDNA3/Hygro vectors (Invitrogen) containing the coding sequence of human K_v1.2 (hK_v1.2) channel or human K_{Ca}2.3 (hK_{Ca}2.3) channel, were co-transfected together with a GFP construct. Currents were measured 1–3 days after transfection. In all cases, FuGene6 Transfection Reagent (Roche, Mannheim, Germany) was used for transfection, according to the recommended protocol.

Electrophysiological recordings

All experiments were carried out at room temperature (22–25°C) using the whole-cell recording mode of the patch-clamp technique [12,13]. Cells were bathed with mammalian Ringer's solution containing 160 mM NaCl, 4.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂ and 10 mM Hepes, pH 7.4 (with NaOH), with an osmolality of 290–320 mOsm. When OSK1 peptides were applied, 0.1% (w/v) BSA was added to the Ringer's solution. A simple syringe-driven perfusion system was used to exchange the bath solution in the recording chamber. The internal pipette solution used for measuring voltage-gated K⁺ currents contained 155 mM KF, 2 mM MgCl₂, 10 mM EGTA and 10 mM Hepes, pH 7.2 (with KOH), with an osmolality of 290–320 mOsm. For measuring K⁺ currents through K_{Ca}3.1 and K_{Ca}2.1–2.3 channels, an internal pipette solution containing 135 mM potassium aspartate, 8.7 mM CaCl₂, 2 mM MgCl₂, 10 mM EGTA and 10 mM Hepes, pH 7.2 (with KOH), with an osmolality of 290–320 mOsm {free [Ca²⁺]_i (internal calcium concentration) = 10^{−6} M}, was used. Currents through mK_v1.1, hK_v1.2, hK_v1.4, hK_v1.5, mK_v1.7 and mK_v3.1 channels were elicited with depolarizing voltage steps from −80 mV to +40 mV, for 200 ms applied every 10 s. For recordings from mK_v1.3 channel, the same pulse step was applied every 30 s. Currents through hK_{Ca}3.1, hK_{Ca}2.1, rK_{Ca}2.2, hK_{Ca}2.3 and hK_{Ca}1.1 (hBK or hSlo α) channels were elicited by 1 μ M [Ca²⁺]_i. For SK and K_{Ca}3.1 channels, a voltage-ramp from −120 mV to 0 (K_{Ca}3.1) or +40 mV of 400 or 200 ms duration was used, while K_{Ca}1.1 currents were recorded with a voltage-ramp from −100 to +100 mV (200 ms) applied

every 10 s. Electrodes were pulled from glass capillaries (Science Products, Hofheim, Germany) in three stages, and fire-polished to resistances measured in the bath of 2.5–5 MΩ. Membrane currents were measured with an EPC-9 or EPC-10 patch-clamp amplifier (HEKA Elektronik, Lambrecht, Germany) interfaced to a Macintosh or PC running acquisition and analysis software (Pulse and PulseFit). When voltage-dependent K⁺ currents were measured, the capacitive and leak currents were subtracted using the P/10 procedure. Series resistance compensation (> 80 %) was employed if the current exceeded 2 nA. The holding potential in all experiments was –80 mV. Data analyses were performed with IgorPro (WaveMetrics, Lake Oswego, OR, U.S.A.), and K_d values were deduced by fitting a modified Hill equation to the data {X_{toxin}/X_{control} = 1/[1 + ([toxin]/K_d)], where X is the peak current (for K_v channels), or the slope of the ramp current, i.e. the conductance, measured between –100 and –60 mV (for hK_{Ca}3.1 and hK_{Ca}2.3 channels) to the normalized data points obtained at more than four different peptide concentrations. This fit indicates that one peptide molecule is sufficient to block the current through the channel. The S.D.s obtained by this fitting routine reflect the uncertainty of the fit.

Synthesis of OSK1 and its analogues

OSK1 exhibits a threonine residue at position 36, where most other several toxin families (α -KTx1, α -KTx2, α -KTx4, α -KTx6 and α -KTx12) display a tyrosine residue. Since the latter residue has often been involved in K⁺ channel interaction as part of toxin 'functional' dyads [2], we therefore expected that Thr³⁶ would influence the pharmacology of OSK1 towards K⁺ channels. Accordingly, we produced OSK1 chemically and a series of OSK1 analogues to assess the importance of the specific OSK1 residues Arg¹², Glu¹⁶, Lys²⁰ and Thr³⁶ (Figure 1b). All peptides were prepared by solid-phase synthesis using the stepwise Fmoc/*t*-Bu chemistry as described in [14]. In all cases, the amounts of target peptides linked to their resins illustrate yields of peptide assemblies ranging from 70 % to 80 %. Accordingly, crude reduced peptides, obtained after TFA treatment, were found to be relatively homogeneous, as shown for OSK1 synthesis (Figure 2a). The peptides were folded/oxidized under alkaline conditions for 40–120 h (depending on the nature of the molecule), yielding the crude oxidized peptides (see Figure 2b for OSK1). The main oxidized species were purified to > 99 % homogeneity by preparative C₁₈ reversed-phase HPLC (Figure 2c for OSK1 is shown as an example). The mass spectrometric analyses of the peptides using the MALDI-TOF technique gave experimental molecular masses that are similar to or identical with the deduced molecular masses (Figure 2d). The yield of each peptide synthesis was approx. 1–3 %. In the case of OSK1, a complete Edman sequencing was also performed to validate its primary structure (results not shown) and check for the presence of standard half-cystine pairings of the type C1–C4, C2–C5 and C3–C6, common to three-disulphide-bridged toxins structured according to an α/β scaffold [1,15].

The two-dimensional ^1H -NMR spectrum of synthetic OSK1 is consistent with a peptide folding according to an α/β scaffold, with evidence for α -helical and β -sheet structures (Figure 3a). These experimental data are consistent with the 3D solution

Figure 2 Synthesis and characterization of OSK1 and its analogues

(a) Analytical C₁₈ reversed-phase HPLC elution profile of crude reduced OSK1. Note the presence of non-peptide chemical scavengers in the elution profile (elution times approx. 15 min) that later 'disappeared' during oxidative folding, presumably by sticking to the plastic support. (b) Elution profile of crude folded/oxidized OSK1 after 72 h oxidation time. (c) Elution profile of folded/oxidized OSK1 after purification. (d) Deduced and experimental relative molecular masses (M+H)⁺ of synthetic OSK1 and analogues.

structure of the natural OSK1 solved by ¹H-NMR [1]. We also used one-dimensional ¹H-NMR to verify that the mutations introduced in the OSK1 analogues did not alter folding according to the α/β scaffold (Figure 3b). Indeed, similar overall distributions of the resonance frequencies of the different peptides indicate that they adopt closely related conformations.

Lethal activity of OSK1 and its analogues in mice

We determined LD₅₀ values for the synthetic peptides by injecting them intracerebroventricular into mice (Table 1). All peptides proved to be lethal in mice with symptoms (tremors, convulsions and spasmic paralysis) resembling those induced by inoculation of K⁺ channel toxin blockers (S. Mouhat and J.-M. Sabatier, unpublished work). The order of *in vivo* toxicity was OSK1 > [K₁₆,D₂₀]-OSK1 > [K₁₆]-OSK1 > [D₂₀]-OSK1 > [P₁₂,K₁₆,D₂₀]-OSK1 > [K₁₆,D₂₀,Y₃₆]-OSK1. The greatest difference observed in LD₅₀ values was 4.5-fold between OSK1 and [K₁₆,D₂₀,Y₃₆]-OSK1. The LD₅₀ value observed for OSK1 is of the same order as the LD₅₀ values observed for other potent K⁺-channel-acting scorpion toxins [15–17]. Assuming that OSK1 would diffuse immediately throughout the entire mouse brain (40 ng of toxin in 400 μ l of brain volume) following the injection, the toxin concentration in the brain would be approx. 20 nM. Right at the injection site, the toxin might even briefly

reach micromolar concentrations. We therefore believe that the convulsions and the lethality of the OSK1 peptides are caused by inhibition of brain K_v1.1 and K_v1.2 channels (see IC₅₀ values for these channels in Table 1 and the following section).

Pharmacological activity of OSK1 and its analogues on various K⁺ channels

Effects of OSK1

We studied the pharmacological profile of OSK1 on a panel of 14 K⁺ channels (nine voltage-gated and five Ca²⁺-activated K⁺ channels). Table 1 illustrates that OSK1 is a highly potent K_v channel blocker. The most remarkable features of OSK1 are: (i) its potency to block K_v1.3 channel at picomolar concentrations (IC₅₀ value of 14 pM), which classifies it among the best K_v1.3 channel blockers (with *Stichodactyla helianthus* sea anemone ShK toxin, and AgTx2 and *Heterometrus spinnifer* HsTx1 scorpion toxins [5,18–20]), and (ii) its ability to block all three K_v channels (K_v1.1, K_v1.2 and K_v1.3) with high potency. The decreasing order of blocking efficacy is K_v1.3 > K_v1.1 > K_v1.2 (lower IC₅₀ value of 5.4 nM). This ability to block all three K⁺ channels is unusual, since most toxins characterized so far are either active on K_v1.2 or K_v1.3 channel alone, or on the combination of K_v1.1 and K_v1.3 channels [14,15,19–24]. This peculiar behaviour of OSK1 does not stem from a lack of specificity, since the toxin is inactive

Figure 3 Structural analysis of OSK1 and its analogues by ¹H-NMR

(a) Contour plot of a NOESY spectrum of synthetic OSK1. Fingerprint and amide proton regions are shown on top and bottom, respectively. (b) One-dimensional ¹H-NMR spectra of OSK1 and its analogues. Only the representative amide proton regions are shown. Note that the overall distribution of resonance frequencies suggests that the 3D structures of all six peptides are similar.

Table 1 Bioactivities of OSK1 and its analogues *in vivo* in mice and *in vitro* on 14 different K⁺ channel types

LD₅₀ values for each toxin are given in parentheses (μg/kg of mice). See the Experimental section for details. No effect (ne) was seen for any of the peptides on K_v1.4, K_v1.5, K_v1.6, K_v3.1, K_v11.x, K_{Ca}2.1, K_{Ca}2.2, K_{Ca}2.3 and K_{Ca}1.1 channels, indicating a lack of blocking activity at micromolar peptide concentration.

Channel	IC ₅₀ (nM)					
	OSK1 (2)	[K ₁₆]-OSK1 (3)	[D ₂₀]-OSK1 (4.5)	[K ₁₆ ,D ₂₀]-OSK1 (2.5)	[P ₁₂ ,K ₁₆ ,D ₂₀]-OSK1 (7.5)	[K ₁₆ ,D ₂₀ ,Y ₃₆]-OSK1 (9)
K _v 1.1	0.60 ± 0.04	0.63 ± 0.05	2.95 ± 0.24	0.40 ± 0.01	3.18 ± 0.11	34.4 ± 0.3
K _v 1.2	5.40 ± 1.89	5.23 ± 0.22	77.8 ± 9.2	2.96 ± 0.01	196 ± 9	232 ± 11
K _v 1.3	0.014 ± 0.001	0.067 ± 0.006	0.037 ± 0.007	0.003 ± 0.001	0.059 ± 0.003	0.122 ± 0.007
K _v 1.7	ne	ne	ne	ne	ne	1500 ± 500
K _{Ca} 3.1	225 ± 10	151 ± 21	716 ± 10	228 ± 92	2600 ± 400	885 ± 18

on K_v1.4, K_v1.5, K_v1.6, K_v1.7 and K_v11.x channels (Table 1). Also, OSK1 has no effect on K_{Ca}2.1, K_{Ca}2.2, K_{Ca}2.3 and K_{Ca}1.1 channels when in the micromolar concentration range, whereas it shows a moderate activity on K_{Ca}3.1 channel (also referred to as IK1 or SK4) with an IC₅₀ value of 225 nM. Figure 4 summarizes the effects of OSK1 on the channels that are sensitive to this toxin. It illustrates that saturating concentrations of OSK1 induce a complete block of the outward K⁺ current for each channel type. A comparison of the pharmacological properties of synthetic OSK1 with its natural counterpart was not possible since the latter was not available.

Effects of OSK1 analogues

We have noticed that OSK1 displays two unusual properties for a member of the α-KTx3 family: one structural and one functional. First, some charged amino acid residues differ between OSK1 and other α-KTx3 toxins, such as the acidic Glu¹⁶ and the basic Lys²⁰, which are only present in OSK1. At equivalent positions, other toxins have a basic lysine residue and an acidic aspartate residue. One exception to this rule is Bs6 (α-KTx3.8; Figure 1) that possesses an uncharged glutamine residue at position 16. Overall, this corresponds to an inversion of charged residues within the

Figure 4 Block of voltage-gated $K_v1.1$, $K_v1.2$, $K_v1.3$ and Ca^{2+} -activated $K_{Ca3.1}$ channels by OSK1

(a) Original current traces in the absence (control) or presence of synthetic OSK1 at various concentrations. Top left, block of $K_v1.1$ current by OSK1. Top right, block of $K_v1.2$ current. Bottom left, block of $K_v1.3$ current. For each K_v -channel type, currents were elicited by depolarizing voltage steps from the holding potential -80 mV to $+40$ mV for 200 ms. Bottom right, OSK1-induced block of $K_{Ca3.1}$ current. Currents were elicited by 1μ M $[Ca^{2+}]$, and voltage ramps from the holding potential -80 mV to $+40$ mV for 400 ms. (b) Average normalized current inhibition by various concentrations of OSK1 for each channel type. Results are means $\pm$ S.D. ($n = 4-5$).

OSK1 primary structure. Secondly, OSK1 has an unexpected wide range of activity, i.e. on $K_v1.1$, $K_v1.2$ and $K_v1.3$ channels, a property usually not shared by other toxins from this structural family. We therefore speculated that these two properties might be connected and decided to evaluate the pharmacological impact of restoring the more conventional amino acid residues at relevant positions within the OSK1 sequence (Lys¹⁶ instead of Glu¹⁶, and Asp²⁰ instead of Lys²⁰). First, we investigated the effects of mono-substitutions: $[K_{16}]$ -OSK1 and $[D_{20}]$ -OSK1 (Table 1). For $[K_{16}]$ -OSK1, only slight differences in the pharmacological profile were observed, except the $K_v1.3$ channel, for which a 4.8-fold decrease in blocking efficacy was found (IC_{50} value of 67 pM instead of 14 pM). For $[D_{20}]$ -OSK1, more pronounced effects were observed, with decreased blocking efficacy ranging from 2.5- to 14-fold depending on the ion channel type under consideration (Table 1). Thus both amino acid residues of OSK1 appear to contribute to differences in toxin selectivity for K_v channels. In the

case of OSK1, there is a 385-fold difference in blocking efficacy between $K_v1.2$ and $K_v1.3$ channels, which is decreased to 78-fold for $[K_{16}]$ -OSK1, and increased to 2102-fold for $[D_{20}]$ -OSK1. The two substitutions therefore generated somehow opposite effects in altering toxin selectivity towards K_v channels. We investigated further the potential effects of the double substitution on OSK1 pharmacology. We observed that $[K_{16},D_{20}]$ -OSK1 annulled both tendencies observed with $[K_{16}]$ -OSK1 and $[D_{20}]$ -OSK1, taken individually, such that the pharmacological properties of $[K_{16},D_{20}]$ -OSK1 and OSK1 resembled each other quite closely. However, the blocking efficacy of $[K_{16},D_{20}]$ -OSK1 is increased 1.5-, 1.8- and 4.5-fold on $K_v1.1$, $K_v1.2$ and $K_v1.3$ channels respectively. The difference in blocking efficacy toward $K_v1.2$ and $K_v1.3$ channels is 962-fold, getting closer to what was observed for OSK1, but underlining the greater impact of Asp²⁰ substitution. Importantly, this double substitution generated an OSK1 analogue that is extremely potent on $K_v1.3$ channel, with an IC_{50} value of 3 pM

Figure 5 Activity of OSK1 analogues on K_v channels

(a) Average normalized inhibition of K_v1.1, K_v1.2, K_v1.3 and K_{Ca}3.1 currents by various concentrations of [K₁₆,D₂₀]-OSK1. Each data point corresponds to the mean $\pm$ S.D. ($n = 4-5$). (b) Average normalized inhibition of K_v1.1 currents by various concentrations of [P₁₂,K₁₆,D₂₀]-OSK1 ($n = 5$) and comparison with OSK1 ($n = 5$). (c) Average normalized inhibition of K_v1.2 currents by various concentrations of [P₁₂,K₁₆,D₂₀]-OSK1 ($n = 3$). (d) Average normalized inhibition of K_v1.3 currents by various concentrations of [P₁₂,K₁₆,D₂₀]-OSK1 ($n = 5$). (e) Current traces elicited at +40 mV for control condition or after application of 1 μ M OSK1 ($n = 4$) or 1.5 μ M [K₁₆,D₂₀,Y₃₆]-OSK1 ($n = 5$).

(Figure 5a). This value is similar [5] or even better [19,20] than those reported for the best K_v1.3 channel blockers described hitherto.

Into this most active analogue, we introduced additional substitutions with the aim of determining their impact on OSK1 pharmacology (Figure 1b). First, we replaced Arg¹² of [K₁₆,D₂₀]-OSK1 by a proline residue. The resulting [P₁₂,K₁₆,D₂₀]-OSK1 analogue has decreased activity towards all K⁺ channels tested by factors ranging from 8- to 66-fold as compared with [K₁₆,D₂₀]-OSK1, and from 4- to 36-fold as compared with OSK1 (Figures 5b-5d). Although decreased blocking efficacy is generally not a sought-after property for analogues, it should be noticed that [P₁₂,K₁₆,D₂₀]-OSK1's potency towards K_v1.3 channel remains high (IC₅₀ value of 59 pM), and that the difference in blocking efficacy between K_v1.2 and K_v1.3 channels is increased to 3322-fold. Altogether, these results suggest the importance of Arg¹² for OSK1 pharmacology. However, the pharmacological

impact of the substitution may be due to either the lack of Arg¹² in toxin sequence or a change of peptide conformation induced by Pro¹². Next, we investigated the effect of replacing Thr³⁶ by a tyrosine residue that is found in a homologous position in a number of K_v-channel-acting scorpion toxins from other α -KTx families (Figure 1). This supplemental mutation in [K₁₆,D₂₀]-OSK1 results in a marked decrease of peptide blocking efficacy towards K_v1.1, K_v1.2 and K_v1.3 channels (from 3.9- to 86-fold, and from 8.7- to 57-fold, as compared with OSK1). Interestingly, we found that [K₁₆,D₂₀,Y₃₆]-OSK1 is significantly active towards K_v1.7 channel [25] (Figure 5e), albeit with low potency (IC₅₀ value of 1.5 μ M). It is worth noting that none of the other analogues had gained a novel activity so far towards a previously unblocked channel type. Such a channel blockage is of interest, since there is no known inhibitor of this channel type. Future experiments will aim at improving peptide affinity for K_v1.7 channel.

	S5	Turret region	Pore helix	Selectivity filter	S6
K _{Ca} 3.1	220 -WVLS	VAER-QAVNATGH	LSDTLWLIPIT	FL TIGYGDV VEGTMW	GKIVCL 268
K _v 1.3	366 SSAAY	FAEADDPSSGFNS	IPDAFWWAVVT	MT TVGYGDM HPVTIG	GKIVGS 415
K _v 1.2	343 SSAVY	FAEADERESQFPS	IPDAFWWAVVS	MT TVGYGDM VPTTIG	GKIVGS 392
K _v 1.1	341 SSAVY	FAAEAEAESEHFS	IPDAFWWAVVS	MT TVGYGDM YPVTIG	GKIVGS 390

Figure 6 Comparison of amino acid sequences of pore domains of K_v1.3 with K_v1.1, K_v1.2 and K_{Ca}3.1 channels

Each K⁺ channel amino acid sequence is numbered and subdivided into structural and/or functional regions (transmembrane segments S5 and S6, turret region, pore helix and selectivity filter). Amino acid sequence identity with K_v1.3 channel is shaded grey. Amino acid residues predicted to interact with OSK1 are denoted by asterisks.

DISCUSSION

Although the 3D structure of OSK1 in solution was solved a few years ago [1], its pharmacological properties remained to be established. In the present study, for the first time, we have produced synthetic OSK1, along with several structural analogues, and have investigated their biological activities extensively on a wide range of potential K⁺ channel targets. OSK1 is a potent blocker of three different K_v channel types (K_v1.1, K_v1.2 and K_v1.3) and a moderate blocker of Ca²⁺-activated K_{Ca}3.1 channel. Of interest, OSK1 is one of the most powerful K_v1.3 channel blockers so far described, making it an interesting lead compound for the development of new drugs for the therapy of autoimmune disorders [18,26–29]. A first step in this direction was taken with the production of [P₁₂,K₁₆,D₂₀]-OSK1, which still shows a high affinity, but an improved selectivity for K_v1.3 channel. Also, [K₁₆,D₂₀]-OSK1 exhibits an increased activity towards K_v1.3 channel (IC₅₀ value of 3 pM with approx. 100 % of current block) compared with OSK1, which places it among the most potent K_v1.3 channel blockers reported so far [18]. Therefore [K₁₆,D₂₀]-OSK1 (or its derivatives) might be of particular value for the management of memory-T-cell-mediated immune responses, such as multiple sclerosis [18,29].

In addition, K_v1.3 and K_{Ca}3.1 channels have been shown to critically contribute to lymphocyte activation [18,30]. However, the contribution level of each channel type to lymphocyte K⁺ current is different, depending on the type of activated lymphocytes. K_v1.3 current is predominant in effector memory lymphocytes, whereas K_{Ca}3.1 current predominates in central memory lymphocytes. An ideal immunomodulatory compound is expected to possess two main properties. First, it should preferentially block either K_v1.3 or K_{Ca}3.1 channel in order to target one specific subset of T-cells [18]. Secondly, it should be selective for one of these channel types, thus without affecting other K_v-variants, in order to avoid unwanted secondary effects. OSK1 presents the first desired property, since it targets K_v1.3 channel with a greater potency than K_{Ca}3.1 channel. Also, its lower efficacy on other K_v-type channels, namely the neuronal variants K_v1.1 and K_v1.2 channels, is a first asset towards the design of an adequate immunosuppressive drug. Designing of improved compounds is possible since several OSK1 analogues already display increased selectivity for K_v1.3 channel. It is worth mentioning that OSK1 thus now belongs to an enlarged family of potent K_v1.3 (α -KTx 1.1, 2.2, 3.2 and 7.1) and/or K_{Ca}3.1 (α -KTx 1.1 and 6.2) channel immunosuppressive drugs [31,32]. Forthcoming OSK1-derived analogues will be designed to maintain a high potency on K_v1.3 channel and a diminished activity towards other K_v-channels.

To gain insight in the differences of OSK1 affinity for the various K⁺ channels on which this toxin is active, we first compared the amino acid sequences of the pore regions of K_{Ca}3.1, K_v1.1, K_v1.2 and K_v1.3 channels with a particular emphasis on the last channel type (Figure 6). Noteworthy, K_v-type channels mainly differ in the turret region, whereas amino acid sequence differences with K_{Ca}3.1 channel relies further on the pore helix region. Therefore differences in selectivity may preferentially be attributed to specific interactions of toxin residues with these two regions. In particular, a cluster of acidic residues can be observed in the turret region of K_v-type channels, which is not the case in K_{Ca}3.1 channel. We set out to determine the functional maps of OSK1 on K_v1.1, K_v1.2 and K_v1.3 channels using docking simulations (Figure 7). Of note, the functional map of OSK1 for K_{Ca}3.1 channel could not be formally determined owing to its weak docking energy. According to these functional maps, the channel residues involved in an interaction with OSK1 are highlighted by asterisks (Figure 6). Amino acid residues of the various K_v channels that interact with OSK1 appear to be mainly located in the turret region and in or nearby the selectivity filter. A particular focus on K_v1.3 channel, which is the channel most sensitive to OSK1, reveals the implication of Asp³⁷⁶, Ser³⁷⁸, Asn³⁸², Tyr⁴⁰⁰, Gly⁴⁰¹, Asp⁴⁰², Met⁴⁰³ and His⁴⁰⁴ residues. Of note, His⁴⁰⁴ behaves as a pivotal residue, since it inhibits the effect of α -KTx6.2 (maurotoxin) on K_v1.3 channel [33].

Computational simulations (based on Brownian dynamics) with six other toxins, also highly potent on K_v1.3 channel, reveal a slightly distinct pattern of molecular interactions [34]. In particular, two channel residues (Gly³⁸⁰ and Asp³⁸⁶) were predicted to participate in the interaction with these toxins unlike with OSK1. This suggests that OSK1 has a different docking position over K_v1.3 channel from those of other K_v1.3-channel-acting toxins. Importantly, none of the amino acid residues (Arg¹², Glu¹⁶, Lys²⁰ and Thr³⁶) that were mutated to produce the OSK1 analogues are directly implicated in binding to K_v-type channels. For most of these residues (Arg¹², Glu¹⁶ and Lys²⁰), this was expected, since they belong to the toxin helical structure. Nevertheless, mutating these amino acid residues presumably alters the structure of OSK1, since the docking of [K₁₆,D₂₀]-OSK1 presents a slightly different pattern of molecular contacts, although the same toxin residues are concerned. In particular, [K₁₆,D₂₀]-OSK1 now also interacts with Gly³⁸⁰, similar to other K_v1.3-channel-blocking toxins, but different from OSK1 [34]. Globally, these changes in receptor–ligand contacts may be responsible for the increased affinity of [K₁₆,D₂₀]-OSK1 for K_v-type channels (especially K_v1.3) over OSK1. In that respect, Gly³⁸⁰ may be one of the contributors of this increased affinity, since it is present in K_v1.3, but not in K_v1.1 or K_v1.2, channel.

Figure 7 Functional maps of OSK1 on to K_v1.1, K_v1.2 and K_v1.3, and of [K₁₆,D₂₀]-OSK1 on to K_v1.3 channels

Theoretical functional maps of OSK1 and [K₁₆,D₂₀]-OSK1 depicting amino acid residues that are key for peptide interaction with K_v1.1, K_v1.2 or K_v1.3 channels. Interacting residues from the K_v channel are highlighted in red. I to IV before channel residue numbering specifies one of the four α -subunits forming a functional K_v channel. Colour codes: yellow (hydrophobic residues), light green (polar residues) and blue (basic residues). These maps were generated by docking simulations [37]. Swiss-Prot accession codes used are P16388 (mouse K_v1.1 channel), P16389 (human K_v1.2 channel) and P16390 (mouse K_v1.3 channel).

Although the docking simulation of OSK1 on the K_{Ca}3.1 channel was not reliable, there are interesting parallels to draw from the comparative effects of other toxins on both K_v1.3 and K_{Ca}3.1 channel. Maurotoxin is highly active on K_{Ca}3.1 channel, but is weakly active on K_v1.3 channel [35]. Conversely, OSK1 is weakly active on K_{Ca}3.1 channel, but is highly potent on K_v1.3 channel. Thus a comparison of the functional maps of both toxins should provide some insights into the key molecular determinants that drive these differences in channel selectivity. All functional maps are not available, but a few elements help to identify these molecular determinants. We have noticed that K_v1.3 and K_{Ca}3.1 channels differ mainly in the turret and pore helix regions. However, since docking simulation of OSK1 on to K_v1.3 channel highlights a number of close contacts only in the turret region, this suggests that the latter region may underlie the difference in OSK1 affinity for K_v1.3 and K_{Ca}3.1 channels. In agreement with this hypothesis was the finding that

mutating Lys³² of α -KTx1.1 (charybdotoxin), a residue which interacts with Asp³⁷⁶ of the K_v1.3 channel turret region, strongly decreased toxin affinity for K_v1.3 channel without altering affinity for K_{Ca}3.1 channel [36]. An equivalent lysine residue is present in OSK1 (Lys³²), which interacts with Asp³⁷⁶ from K_v1.3 channel. We thus expect that the higher affinity of OSK1 for K_v1.3 over K_{Ca}3.1 channel is also partly due to this interaction in the turret region. Although helpful, structural considerations from docking simulations are theoretical, and further experimental validation through targeted point mutations will be needed to confirm the functional importance of the turret region in underlying differences in toxin selectivity.

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