*Pure & Appl. Chem.*, Vol. 68, No. 11, pp. 2105–2109, 1996.
Printed in Great Britain.


# Structure and function of the potassium channel inhibitor from black scorpion venom

E.V.Grishin, Yu.V.Korolkova, S.A.Kozlov, A.V.Lipkin, E.D.Nosyreva, K.A.Pluzhnikov, S.V.Sukhanov, T.M.Volkova

Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, ul. Miklukho-Maklaya, 16/10, 117871, GSP-7, Moscow, Russia

***Abstract***
A novel inhibitor of $K^+$ channels has been purified from the venom of the Central Asian scorpion *Orthochirus scrobiculosus*. For this polypeptide toxin (OsK-1) with molecular mass 4205.7 Da complete amino acid sequence was determined by Edman degradation and C-terminal amino acid analysis, and was confirmed by cloning and sequencing of the toxin cDNA. OsK-1 consists of 38 amino acid residues and possesses high sequence homology with agiotoxin, kaliotoxin and some homology with other known $K^+$-channel blockers from different scorpion venoms. The toxin was shown to block small-conductance $Ca^{++}$-activated $K^+$-channels in neuroblastomaxglioma NG 108-15 hybrid cells ($K_d$ =1.4 x $10^{-7}$ M) which are insensitive to apamin and sensitive to charybdotoxin. The effect of OsK-1 was reversible and concentration dependent.

## INTRODUCTION

Natural venoms are a rich source of molecules that interact with membrane receptors and ionic channels. Due to peptide toxins derived from venoms of a variety of invertebrates and lower vertebrates, valuable information about mechanisms of neurotransmission, properties and physiological role of voltage-dependent $Na^+$,$Ca^{++}$ and different types of $K^+$-channels has been obtained.

Potassium channels comprise a large, diverse group of integral membrane proteins which are involved in cell volume regulation, mediation of hormonal secretion, and determination of level of excitability and repolarization properties of neurons and muscle fibres. On the basis of gating mechanisms $K^+$-channels can be subdivided into at least two distinct classes: voltage-activated $K^+$-channels respond to changes in membrane potential, whereas gating of $Ca^{++}$-activated $K^+$-channels is dependent on intracellular $Ca^{++}$, although members of the second class are also sensitive to voltage. Within the latter class of channel variation in calcium sensitivity, voltage-dependence of channel opening, single channel conductance and sensitivity to blockers have been observed, suggesting that there may be subtypes of closely related $Ca^{++}$-activated $K^+$-channels (1,2,3,4).

In spite of intensive investigation in recent years, up to date knowledge about the majority of potassium channel subtypes is not complete. In many cases the lack of information has been at least counterbalanced by numerous data based on an electrophysiological approach combined with the use of specific blockers. The main sources for obtaining of $K^+$-channel blockers (toxins) are venoms of honey bees, scorpions and snakes. Such toxins as apamin, which blocks small-conductance $Ca^{++}$-activated $K^+$-channels, charybdotoxin (ChTX) - a blocker of high conductance $Ca^{++}$-activated $K^+$-channels and certain voltage-dependent $K^+$-channels as well, dendrotoxin - a blocker of voltage dependent $K^+$-channels, are traditionally used for identification and differentiation of $K^+$-channels. At present a family of $K^+$-channel toxins is replenished mainly at the expense of scorpion toxins. New classes of toxins are useful in developing the molecular phar-

macology of potassium channels. The present paper is devoted to the isolation and study of a novel toxin from venom of the scorpion *Orthochirus scrobiculosus,* which inhibits the small-conductance apamin resistant $Ca^{++}$-activated $K^+$-channel.

## RESULTS

Crude venom obtained from the Central Asian scorpion *Orthochirus scrobiculosus* was tested on different types of potassium channels, namely potential dependent (delayed rectifier type) and $Ca^{++}$-activated channels of different conductance. It was shown that crude venom possesses inhibitory activity on both types of channels.

To get rid of mucines, solution of a crude venom was subjected to continuing centrifugation and filtration through a 0.45 μm membrane. The obtained solution was separated by size-exclusion chromatography on a TSK-2000 SW HPLC column (7.5 x 600 mm) in 0.05 M ammonium acetate buffer (pH 5.7) containing 0.15 M NaCl. All chromatographic fractions were tested for inhibitory activity on different types of $K^+$-channels. The fraction containing polypeptides with molecular masses 4-12 KDa was active on $Ca^{++}$-activated $K^+$ small-conductance channels. This fraction was further purified by RP - HPLC in 0.1% trifluoroacetic acid (Fig. 1).

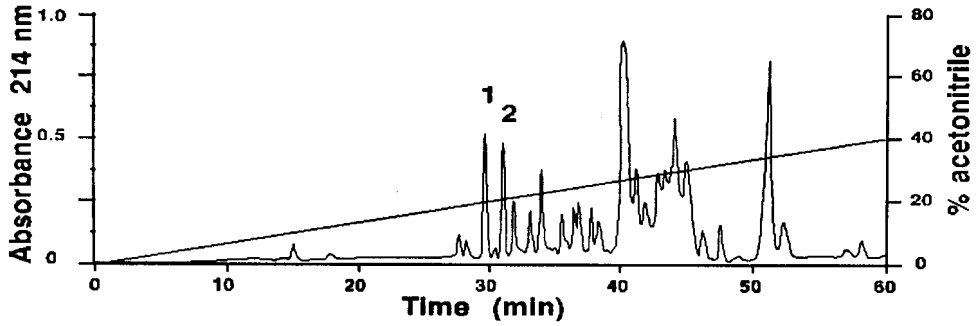


Fig. 1 Chromatography on a RPMC column (4.6 x 250 mm) of active fraction obtained by size-exclusion fractionation. Flow rate 1ml/min, gradient of acetonitrile concentration.

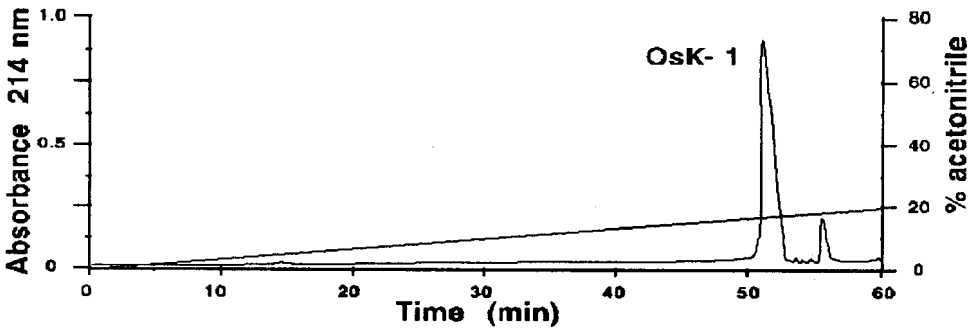


Fig. 2 Rechromatography of fraction 1 (Fig.1) on an Ultrasphere ODS column (4.6 x 250 mm) in a gradient of acetonitrile concentration, flow rate 1 ml/min.

Only fractions 1 and 2 eluted at approximately 20% of acetonitrile concentration (Fig. 1) revealed inhibitory activity on these $K^+$-channels. From fraction 1 accounting for approximately

1% of total weight of *Orthochirus scrobiculosus* venom, polypeptide toxin OsK-1 was isolated by rechromatography on an Ultrasphere ODS column in 0.1% trifluoroacetic acid (Fig. 2). The purity of OsK-1 was shown by N-terminal amino acid analysis (5) and by laser desorption mass spectrometry. Its molecular mass was estimated as 4205.7 Da.

The total amino acid sequence of the protein was established by sequencing on a gas-phase automatic sequenator as well as by C-terminal amino acid analysis. OsK-1 was determined as a single chain protein containing 38 amino acid residues.

The amino acid sequence of OsK-1 was further confirmed by cloning and sequencing of the toxin cDNA by PCR. The first strand cDNA was synthesized using poly $(A)^+$-rich RNA prepared from scorpion telson total RNA (6) by two cycle chromatography on oligo (dT)-cellulose (7). The RACE outer-inner(dT) primer - RoRi(dT) (8) was used for the first strand cDNA synthesis. The RACE-PCR procedure was employed on the first strand cDNA using the degenerate forward primer based on the N-terminal OsK-1 peptide ($Gly_1$ - $Lys_7$) and the RACE outer primer - Ro. A PCR-amplified product of 320 bp was subcloned in pBluescript and sequenced. No difference was observed between the amino acid sequence deduced from cDNA and that determined by protein chemistry methods.

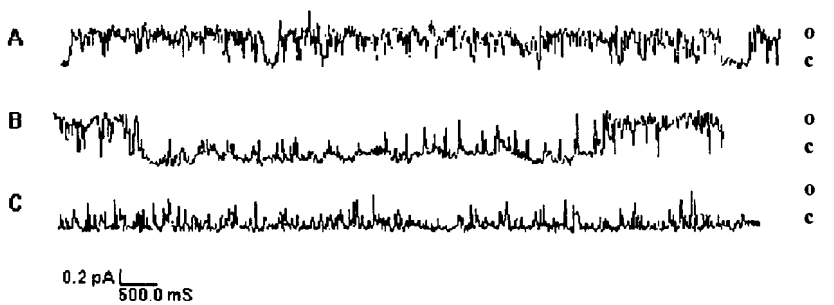


Fig. 3 Single channel currents recorded from an outside-out patch: A - before application; B - after application of 1μM fraction 2; C - after application of 2 μM fraction 1. Holding potentials 0 mV, filtering 0.3 KHz, sampling 3 KHz.

Small-conductance $K^+$-channel with a single channel current rapidly flickering during bursts of activity separated by silent interburst periods was identified from single channel recordings on neuroblastomaxglioma NG 108-15 cells using standard patch-clamp procedures (9). Outward currents were recorded from outside-out patches in asymmetrical conditions (150 mM $K^+$ in /5 mM $K^+$ out) (Fig. 3). The single channel current-voltage relation was found to be linear in the voltage range from -50 mV to +60 mV. The extrapolated reversal potential (-80 mV) was near the calculated equilibrium potential measured for $K^+$-ions (-86 mV) (Fig. 4, ser. 1). Single channel conductance in asymmetrical conditions was about 9 pS. Under symmetrical conditions (150 $K^+$ in / 150 $K^+$ out) single channel conductance was larger for inward currents (about 30 pS in the range -100 - 0 mV) and smaller

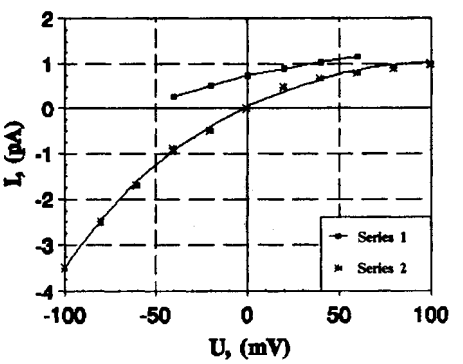


Fig. 4 Current-voltage relations obtained from an outside-out patch.

for outward currents under the same conditions (Fig. 4, ser. 2). The activity of small-conductance $K^+$-channels was independent on membrane potential between -50 and 50 mV and dependent on internal $Ca^{++}$. This dependence was analyzed in inside-out patches at +40 mV. The concentration of free $Ca^{++}$ was adjusted with EGTA or HEEDA using stability constants taken from (10). The channel was active with 1 μM [$Ca^{++}$] and almost totally inactive with 100 nM [$Ca^{++}$] at the inner face of the membrane. The activity of the channel was reversibly inhibited by the addition of 0.5 mM EGTA to the cytosolic membrane side. The channel activity was unaffected by extracellular application of 5 μM of apamin, and was totally and reversibly inhibited by extracellular application of 50 nM of ChTX. The ChTX concentration producing half-maximal inhibition ($IC_{50}$) of the small-conductance $K_{Ca}$-channels was 10 nM. The parameters of the channel in NG 108-15 cells were in agreement with those previously reported (11). The activity of the channel recorded from outside-out patches was reduced after external application of fraction 1 (Fig. 3b) and fraction 2 (Fig. 3c). The inhibitory effect was observed in all the patches tested (n = 5 and n = 2 respectively) and was reversible and concentration dependent.

Application of fraction 1 and 2 caused reduction of single current amplitude and conductance and a decrease of the open state probability ($P_o$), which was calculated from all-points histograms. $K_d = 1.4 \times 10^{-7}$ M for OsK-1 was estimated according to the Hill equation.

## DISCUSSION

OsK-1, a novel blocker of the $K^+$-channel, isolated from *Orthochirus scrobiculosus* scorpion venom, is a single chain, highly positively charged (7 Lys, 2 Arg, against 1 Glu residue) polypeptide consisting of 38 amino acid residues. Comparison of amino acid sequences of OsK-1 with the sequences of known $K^+$-channel blockers from different scorpion venoms shows significant homology (12, 13, 14) (Fig. 5). OsK-1 as well as other blockers contains the 6 conserved half-cysteins and a number of conserved amino acid residues in C-terminal parts of molecule. OsK-1 indicated the high homology with the kaliotoxins group: 79% amino acid residues of OsK-1 are identical to those of kaliotoxin, which specifically blocks the intermediate conductance $Ca^{++}$-activated $K^+$-channels and also binds to the dendrotoxin sensitive voltage dependent $K^+$-channels (15). At the same time the highest degree of sequence homology (82%) is observed between OsK-1 and agiotoxin (AgTX-1) - a high affinity inhibitor of the Shaker $K^+$-channels (14). The lower degree of homology (37%) is found with ChTX, which is clearly non-selective for the $K_{Ca}$-channels. ChTX belongs to a family of toxins which blocks some voltage-activated and dendrotoxin sensitive $K^+$-channels (Shaker $K^+$-channels), but its affinity ($K_i$ = 227 nM) is much lower than the affinity of AgTX ($K_i$ < 1 nM) (14).

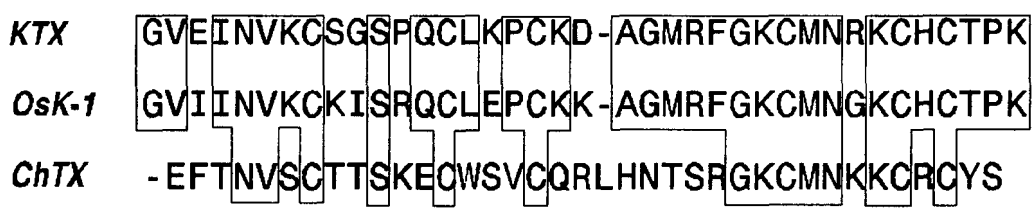


Fig. 5 Amino acid sequence alignment between $K^+$-channel inhibitors.

The modes of action of ChTX and OsK-1 on small-conductance apamin resistant $K_{Ca}$-channels are similar. The effect of both toxins is concentration dependent and reversible. They cause the decrease of channel open state probability. On the other hand the affinity of OsK-1 for this class of channels is less than that of ChTX. This fact is probably due to low homology of OsK-1 and ChTX. High homology of OsK-1 with AgTX makes it possible to suggest that OsK-1 would effectively block Shaker $K^+$-channels. It is interesting to mention the peculiarity of the OsK-1 amino acid sequence. In a central part of kaliotoxin and agiotoxin molecules there are $Lys_{16}$ and $Asp_{20}$ whereas in the OsK-1 molecule one can see $Glu_{16}$ and $Lys_{20}$. Such replacement of charged amino acid residues may play a definite role in specificity of OsK-1 action.

## ACKNOWLEDGMENTS

This work was supported by the Russian foundation of Fundamental Research and International Science Foundation.

We wish to acknowledge the help of Dr. Chertova E.N. for amino acid sequence determinations.

## REFERENCES

1. B. Hille, *Ionic Channels of Excitable Membranes,* ch. **5**, Sinauer Accociates Inc. Publishers Sunderland, Massachusetts (1984).
2. R. Latorre *et al. Annu. Rev. Physiol.* **51**, 385-399 (1989).
3. P.H. Reinhart *et al. Neuron* **2**, 1031-1041 (1989).
4. A.L. Blatz and K. L. Magleby, *Trends Neurosci.* **10**, 463-467 (1987).
5. E. Gross, *Methods of Enzymology* **v.11**, pp. 238-254, Academic Press, Inc. (London) LTD (1967).
6. N. Kiyatkin *et al., FEBS Lett.* **270**, 127-131 (1990).
7. H. Avin and P. Leder, *Prot. Nat. Acad. of Sci.***69**, 1408-1413 (1972).
8. M.A. Frohman *et al. P ot. Nat. Acad. of Sci.* **85**, 8998-9002 (1988).
9. O.P. Hamill *et al. Pflügers Arch.***391**, 85-100 (1981).
10. A. Fabiato and F. Fabiato, *J. Phisiol.,Paris* **75**, 463-505 (1979).
11. C. Van Renterghem and M. Lazdunski, *Pflügers Arch.***420**, 417-423 (1992).
12. G. Giminez-Gallego *et al. Proc. Natl. Acad. Sci.***85**, 3329-3333 (1988).
13. R. Romi *et al. J. Biol. Chem.* **268**, 26302-26309 (1993).
14. M.L. Garcia *et al. Biochemistry* **33**, 6834-6839 (1994).
15. F. Laraba-Djebari *et al. J. Biol. Chem.* **269**, 32835-32843 (1994).