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An ATPase inhibitory peptide with antibacterial and ion current effects

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ABSTRACT

An 84-residue bactericidal peptide, PSK, was purified from a *Chrysomya megacephala* fly larvae preparation. Its amino acid sequence is similar to that of a previously reported larval peptide of the *Drosophila* genus (SK84) noticed for its anticancer and antimicrobial properties. The PSK sequence is also homologous to mitochondrial ATPase inhibitors from insects to humans (35–65% sequence identity), indicating an intracellular protein target and possible mechanism for PSK. It contains a cluster of six glycine residues, and has several two- and three-residue repeats. It is active against both Gram-positive and Gram-negative bacteria via a mechanism apparently involving cell membrane disintegration and inhibition of ATP hydrolysis. In addition, PSK induces an inward cationic current in pancreatic β cells. Together, the findings identify a bioactive peptide of the ATPase inhibitor family with specific effects on both prokaryotic and mammalian cells.

1. Introduction

Flies live in microbial environments where survival requires efficient protective mechanisms [1,2]. In a search for antimicrobial peptides from fly larvae of *Chrysomya megacephala*, we isolated and characterized a multifunctional peptide (PSK, Peptide with terminal Ser and Lys residues) revealing sequence homology with mitochondrial ATPase inhibitors. A similar peptide in *Drosophila virilis* has previously been deduced by cDNA cloning and was noticed for anticancer and antibacterial effects [3], but not for the ATPase inhibitor connection. Multifunctionality has been demonstrated for several peptides of mammalian and insect origins, including sapecin B of *Sarcophaga peregrina* (flesh fly) [4] that has both bacteriolytic and regulatory roles, the latter on larval brain ion (potassium) currents.

ATPase inhibitors are heat-stable, ~90-residue polypeptides playing a regulatory role for ATP synthase activity [5,6]. The binding of these proteins to the ATP synthase F_1 subunit is pH-dependent: below neutrality (approximately pH 6.5), the inhibitory factor is dimeric and active, forming a stable complex with the enzyme, while above neutrality, it forms an inactive tetramer [6]. The active dimers are formed via an antiparallel α-helical coiled-coil interaction in the C-terminal segment (a feature now detected for the PSK sequence), while the inactive tetramers are formed via coiled-coil interactions in the N-terminal, inhibitory region, preventing the binding of the inhibitory factor to ATP synthase [7,8]. About a dozen members of the ATPase inhibitor family have been described and annotated (UniProtKB/Swiss-Prot), and in recent years, antibacterial peptides of both insect and amphibian origins have been shown to influence ATPase activity in bacterial cells [9,10], which is in line with the present results.

2. Materials and methods

2.1. Peptide purification

A preparation of *C. megacephala* fly larvae (5 kg) was used to produce a concentrate of thermostable polypeptides [11]. The concentrate (16 g wet) was dissolved in 0.2 M acetic acid (containing mercaptoethanol), centrifuged, and the supernatant fractionated on Sephadex G–25 (fine), as monitored by antibacterial screening (*Bacillus thuringiensis*). Active fractions were lyophilized, dissolved in 0.01 M ammonium bicarbonate (pH 8.0), and further purified on a CMC 23 column by step-wise increments to 0.01, 0.02, 0.05, 0.1 and 0.2 M ammonium bicarbonate. The fraction at

Abbreviations: CFU, colony forming units; HEK, human embryo-kidney; MBC, minimum bactericidal concentration; PSK, Peptide Ser Lys.

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Table 1
PSK minimum bactericidal concentration (MBC) values.

Bacteria tested	MBC* (μg/μl)
Gram-positive	
Bacillus thuringiensis	0.078
Bacillus subtilis	0.039
Staphylococcus aureus	0.078
Gram-negative	
Pseudomonas aeruginosa	1.2
Escherichia coli	5.0

* Viability test after serial dilution as given in the Section 2 (each concentration level ascertained in five experiments).

0.01 M revealed strong antibacterial activity and was subjected to reverse-phase HPLC on TSK ODS–C18 in 0.1% TFA at 4 ml/min using a linear gradient of acetonitrile (20–50% in 30 min). The active fraction from this step was further purified by reverse-phase HPLC on Vydac 218 TP54 C18 at 1 ml/min (0.1% TFA and 20–25% acetonitrile in 5 min followed by 25–35% in 20 min).

2.2. Antibacterial assays and morphological observations

Antibacterial activity was recorded using an inhibition zone assay [12]. Peptide samples dissolved in water were loaded in 3 mm wells, incubated overnight at 37 °C, and the diameter of the inhibition zone was determined [12]. Minimum bactericidal concentration (MBC, see Table 1) was determined for test organisms from the American Type Culture Collection cultivated in suspension and diluted to 2×10^6 colony forming units (CFU) / ml with sterile saline. Peptide samples dissolved in water (10 μg/μl) were diluted in steps with factors of two (from 5 to 0.009 μg/μl) and aliquots (10 μl) were added to the same volume of bacterium suspension and incubated at 37 °C for 60 min followed by seeding in a sterile agarose plate containing the appropriate medium. The lowest peptide concentration applied without visible bacterial clone after overnight incubation at 37 °C was taken as the MBC. Bacterial surfaces were investigated with and without exposure to PSK using scanning electron microscopy (Hitachi X–650).

2.3. Structural characterization of PSK

Active material from reverse-phase HPLC (above) was applied to Tris-Tricine–SDS–PAGE followed by Coomassie staining. The band with an apparent molecular mass of 9 kDa was electroblotted onto a polyvinylidene difluoride membrane (Millipore) and submitted to sequence analysis by N-terminal degradation (Procise HT, Applied Biosystems) for 40 residues. Overlapping peptides for complete sequence determination were generated by trypsin or GluC specific protease digestion.

2.4. Cell preparations and electrophysiology

Primary cultures of pancreatic β cells from male Wistar rats were prepared as described [13]. Human embryo-kidney (HEK) 293 cells were cultivated in Dulbeccos modified Eagles medium (Gibco) containing fetal bovine serum and penicillin/streptomycin. Currents were recorded with the whole-cell patch-clamp configuration at room temperature (22–25 °C). In experiments with pancreatic β cells, pipettes were filled with 0.1 mM EGTA (pH 7.4). The extracellular solutions contained 2.6 mM $CaCl_2$ (pH 7.4). For Ca^{2+}-free bath solution, 2.6 mM $CaCl_2$ was replaced with 2.6 mM $MgCl_2$. In experiments with HEK293 cells, pipettes were filled with 3 mM $CaCl_2$ (pH 7.4). Experiments were performed with a patch-clamp amplifier (HEKA Electronics, Germany). Macroscopic records were filtered at 2.9 kHz and digitized at typically 20 kHz. Data were analyzed with Clampfit (Axon Instruments, USA), and SigmaPlot (SPSS, USA) softwares.

3. Results

3.1. Purification of PSK

After initial exclusion chromatography, the fraction with *B. thuringiensis* antibacterial activity was applied to ion-exchange chromatography. Only one fraction was antibacterially active and its major peptide component was recovered after repeated reverse-phase HPLC runs as a homogeneous product (a single symmetrical HPLC peak, and a 9 kDa band in Tris-Tricine–SDS–PAGE), and was applied to antibacterial assays, electrophysiology and structural characterization. Recovery of purified PSK was 1.3 mg/kg dry larvae.

3.2. Structural characterization, sequence comparisons and family assignment

The N-terminal sequence was determined by Edman degradation of the intact peptide (residues 1–40) and of proteolytic fragments (Fig. 1). PSK consists of 84 residues (monoisotopic mass 9426.79 Da) and all 13 glycine residues are located in the N-terminal third, where positions 13–18 form a six-glycine cluster. Several two- and three-residue repeats occur (Fig. 1).

Database searches of the PSK sequence (NCBI BLASTP) revealed a number of homologous sequences from the genus *Drosophila*. The searches also revealed sequence homology to mitochondrial ATPase inhibitors in organisms ranging from *Caenorhabditis elegans*, *Bombyx mori* (domestic silkworm) and *Aedes aegypti* (yellowfever mosquito) to humans (Fig. 1). The sequence determined thus indicates that PSK is a member of the mitochondrial ATPase inhibitor family, and that the *D. melanogaster* isoforms A/B and C, the *D. pseudoobscura* protein, and the *D. virilis* SK84 peptide [3], also belong to this protein family.

Based on these findings, several known or predicted ATPase inhibitors were aligned with the PSK sequence (some are shown in Fig. 1). The mosquito *A. aegypti* protein (a predicted ATPase inhibitor) reveals 65% sequence identity to PSK, the *D. melanogaster* isoform A/B 93%, and the peptides are almost identical in the region 7–47 (PSK numbering) (Fig. 1), and contain a segment with a six glycine repeat (GSGAGKGGGGGG) at residues 7–18, followed by the sequences IREAGG (residues 20–25), YF (residues 38–39), and EQL (residues 45–47). This conservation also extends to the

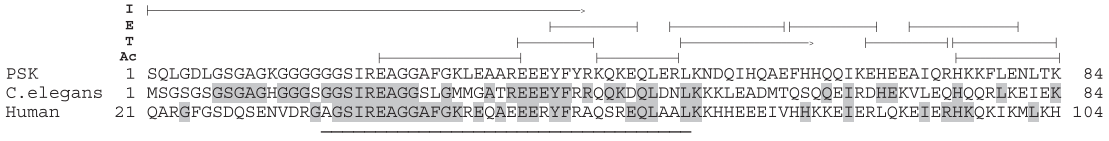

Fig. 1. Determination of the 84-residue PSK primary structure (top sequence). Horizontal lines above the PSK sequence indicate segments covered in separate sequencer runs (I, intact peptide; E, GluC fragments; T, tryptic fragments; and Ac, tryptic fragments generated after substrate acetylation). Also shown is an alignment of PSK with mitochondrial ATPase inhibitory polypeptides from *Caenorhabditis elegans* (NP_508536, 88 residues) and human (Q9UII2, 106 residues), where shading indicating residue identity to PSK. The line at the bottom defines the segment corresponding to the minimal inhibitory sequence of the bovine form [15].

nematode *C. elegans* protein (residues 1–84, 46% identical to the PSK sequence), and to mouse, human (residues 21–104, 39% and 38% identical, respectively), and bovine (residues 1–79, 35% identical) polypeptides (Fig. 1).

The probability of forming an α-helical coiled-coil in PSK is high, between 70 and 100% for residues 30–71 [14]. This is a property known to promote dimerization and oligomerization, and the conserved Ile65 initiates the last heptad repeat in a potential coiled-coil interaction which is a prerequisite for the ATPase inhibitory action [7,8].

3.3. Antibacterial spectrum and effects on bacterial membranes

MBC values show that PSK is more active against G^+ (MBC 0.039–0.078 μg/μl) than G^- bacteria (MBC 1.2–5.0 μg/μl) (Table 1). PSK had no visible effect on yeast when tested at concentrations up to 5.0 μg/μl.

Morphological changes were observed by scanning electron microscopy for a set of bacteria (listed in Table 1) after treatment with PSK. Non-treated bacteria (10^7 CFU / ml) revealed a normal, smooth surface, but already after 5–10 min with PSK (0.020–0.080 μg/μl), disintegration of the bacterial membranes was visible.

3.4. PSK triggers a cation current in pancreatic β cells

Patch-clamp experiments with β cells revealed the formation of an inward cation current during the first 50–100 s after PSK addition (100 nM). After 100 s, a rapid phase of inward current with biphasic components was detected both with and without 2.6 mM Ca^{2+} (Fig. 2A and B). In contrast, no current was detected in control HEK293 cells for over 250 s after addition of PSK (Fig. 2C).

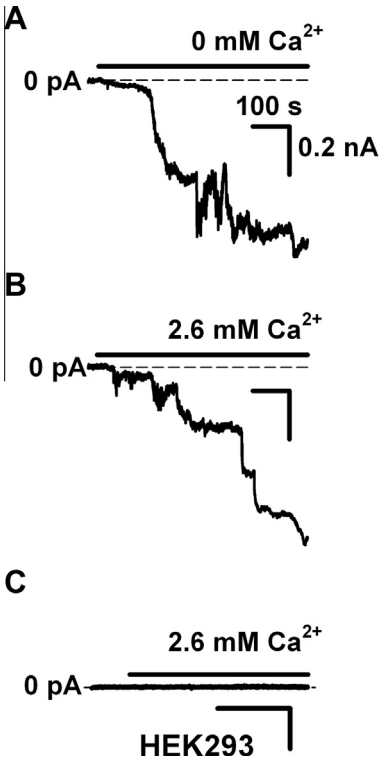

Fig. 2. PSK induces a cation current with biphasic components in pancreatic β cells (A and B), but not in HEK293 cells (C). Horizontal lines: time course after PSK application (100 nM). Scale bars: horizontal, 100 s; vertical, 0.2 nA. Dashed line at 0 pA: baseline. (A and B) Application of PSK to β cells with and without calcium. (C) Application of PSK to HEK293 cells in the presence of calcium. Here the baseline coincides with the current trace (i.e. no current).

4. Discussion

A multifunctional bioactive peptide (PSK) was isolated and characterized from *C. megacephala* larvae, showing an insect member of the mitochondrial ATPase inhibitor family. In addition to disintegration of both G^+ and G^- bacterial membranes, PSK induces a cation current in pancreatic β cells, but not in HEK293 cells. Consequently, the biological effects of PSK are not limited to prokaryotic cells, but also involve mammalian cells in an apparently cell-specific manner. Common to these actions is likely the membrane disintegration potential of PSK.

The bovine mitochondrial ATPase inhibitor has been extensively studied [6–8,15], defining a minimal inhibitory 34-residue sequence for the bovine polypeptide between residues 14–47 [15] (Fig. 1). Translation to PSK reveals that the minimal inhibitory sequence corresponds to residues 19–52, a segment coinciding with the region of extensive sequence conservation.

From structural studies of the bovine ATPase inhibitory polypeptide, it is known that the active form is dimeric, and that dimers are formed through an antiparallel α-helical coiled-coil interaction in the C-terminal region [6]. The PSK sequence displays a high probability for coiled-coil interactions (70–100% in the middle to C-terminal region, residues 30–71), strengthening the conclusion that PSK is a member of the mitochondrial ATPase inhibitor family. In addition, presently available experimental data show that PSK lowers the activity of F_0F_1-ATPase from rat liver mitochondria, an effect probably part of the bactericidal mechanism.

The PSK peptide structure also indicates a relationship to the glycine-rich antibacterial peptide family and our observations reveal that PSK demolishes the viability of G^+ and G^- bacteria within minutes via disintegration of the membrane. The MBC value for PSK against *Staphylococcus aureus* (Table 1) is significantly lower (10–12-fold) than the corresponding values for two antibacterial 32-residue protamines, showing that PSK is a potent agent [16]. Furthermore, there are few reports on peptides active against *B. thuringiensis* [17], while PSK has a significant effect on this bacterium. These findings, and the fact that PSK kills *Pseudomonas aeruginosa*, are of special interest. Together, the data identify a *Chrysomya* peptide, PSK, as an ATPase inhibitor family member with multiple biological actions including antibacterial activity and effects on cellular cation currents.

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References

[1] K.L. Sukontason, M. Bunchoo, B. Khantawa, S. Piangjai, Y. Rongsriyam, K. Sukontason, Comparison between *Musca domestica* and *Chrysomya megacephala* as carriers of bacteria in northern Thailand, Southeast Asian J. Trop. Med. Public Health 38 (2007) 38–44.

[2] A.Z. Sahalan, B. Omar, A.Y. Mohamed, J. Jeffery, Antibacterial activity of extracted hemolymph from larvae and pupae of local fly species, *Musca domestica* and *Chrysomya megacephala*, J. Sains Kesihatan Malaysia 4 (2007) 1–11.

[3] J. Lu, Z.-W. Chen, Isolation, characterization and anti-cancer activity of SK84, a novel glycine-rich antimicrobial peptide from *Drosophila virilis*, Peptides 31 (2010) 44–50.

[4] S.-R. Lee, S. Kurata, S. Natori, Molecular cloning of cDNA for sapecin B, an antibacterial protein of *Sarcophaga*, and its detection in larval brain, FEBS Lett. 368 (1995) 485–487.

[5] B. Frangione, E. Rosenwasser, H.S. Penefsky, M.E. Pullman, Amino acid sequence of the protein inhibitor of mitochondrial adenosine triphosphatase, Proc. Natl. Acad. Sci. USA 78 (1981) 7403–7407.

[6] E. Cabezón, M.J. Runswick, A.G.W. Leslie, J.E. Walker, The structure of bovine IF_1, the regulatory subunit of mitochondrial F-ATPase, EMBO J. 20 (2001) 6990–6996.
[7] E. Cabezón, P.J.G. Butler, M.J. Runswick, J.E. Walker, Modulation of the oligomerization state of bovine F_1-ATPase inhibitor protein, IF_1, by pH, J. Biol. Chem. 275 (2000) 25460–25464.
[8] E. Cabezón, I. Arechaga, P.J.G. Butler, J.E. Walker, Dimerization of bovine F_1-ATPase by binding the inhibitor protein, IF_1, J. Biol. Chem. 275 (2000) 28353–28355.
[9] G. Kragol, S. Lovas, G. Varadi, B.A. Condie, R. Hoffmann, L. Otvos Jr., The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding, Biochemistry 40 (2001) 3016–3026.
[10] T.F. Laughlin, Z. Ahmad, Inhibition of *Escherichia coli* ATP synthase by amphibian antimicrobial peptides, Int. J. Biol. Macromol. 46 (2010) 367–374.
[11] Z.-W. Chen, B. Agerberth, K. Gell, M. Andersson, V. Mutt, C.-G. Östenson, S. Efendic, J. Barros-Söderling, B. Persson, H. Jörnvall, Isolation and characterization of porcine diazepam-binding inhibitor, a polypeptide not only of cerebral occurrence but also common in intestinal tissues and with effects on regulation of insulin release, Eur. J. Biochem. 174 (1988) 239–245.
[12] B. Agerberth, A. Boman, M. Andersson, H. Jörnvall, V. Mutt, H.G. Boman, Isolation of three antibacterial peptides from pig intestine: gastric inhibitory polypeptide (7–42), diazepam-binding inhibitor (32–86) and a novel factor, peptide 3910, Eur. J. Biochem. 216 (1993) 623–629.
[13] Q.-F. Wan, Y.-M. Dong, H. Yang, X.-L. Lou, J.-P. Ding, T. Xu, Protein kinase activation increases insulin secretion by sensitizing the secretory machinery to Ca^{2+}, J. Gen. Physiol. 124 (2004) 653–662.
[14] A. Lupas, M. Van Dyke, J. Stock, Predicting coiled coils from protein sequences, Science 252 (1991) 1162–1164.
[15] M.J. Van Raaij, G.L. Orriss, M.G. Montgomery, M.J. Runswick, I.M. Fearnley, J.M. Skehel, J.E. Walker, The ATPase inhibitor protein from bovine heart mitochondria: the minimal inhibitory sequence, Biochemistry 35 (1996) 15618–15625.
[16] M. Conte, F. Aliberti, L. Fucci, M. Piscopo, Antimicrobial activity of various cationic molecules on foodborne pathogens, World J. Microbiol. Biotechnol. 23 (2007) 1679–1683.
[17] E.G. Abraham, J. Nagaraju, D. Salunke, H.M. Gupta, R.K. Datta, Purification and partial characterization of an induced antibacterial protein in the silkworm, *Bombyx mori*, J. Invertebr. Pathol. 65 (1995) 17–24.