FEBS Letters 347 (1994) 112–116

FEBS 14132

Toxic principle of selva ant venom is a pore-forming protein transformer

A.S. Arseniev, K.A. Pluzhnikov, D.E. Nolde, A.G. Sobol, M.Yu. Torgov, S.V. Sukhanov, E.V. Grishin*

Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Ul. Miklukho-Maklaya 16/10, 117871 Moscow, Russian Federation

Received 22 April 1994

Abstract

Ectatomin (Ea) is a newly isolated main toxic component of *Ectatomma tuberculatum* ant venom. Structural and electrophysiological studies were performed with purified Ea. The protein consists of two homologous polypeptide chains (37 and 34 residues) and forms a four α-helix bundle in aqueous solution. On insertion into artificial bilayer membranes, two Ea molecules form an ion pore. Our results suggest that the 'inside-out' mechanism of pore formation requires a significant movement of Ea helical parts. The pore formation in the cell membrane might well explain the toxic activity of Ea, not excluding at the same time its intracellular activities.

Key words: Ant venom; Ectatomin; Structure; Pore-forming protein; *Ectatomma tuberculatum*

1. Introduction

Considerable progress has been made in analyzing the venoms of selected species of ants [1]. The poison gland products of ants are characterized by large structural diversity and show a wide range of pharmacological activities [1,2].

Here we report the structure and suggest a molecular mechanism of function of an active principle of *Ectatomma tuberculatum* ant venom. This species, which inhabits the Amazonian selva, possesses one of the most toxic venom among ants. This ant subdues its prey by injecting a venom that evacuates through a sting at the tip of the abdomen. The sting is algogenic for human beings and strikes insects and even small animals [3]. The crude venom possesses a high toxicity as measured by lethal dose in male mice after intracerebroventricular (LD_{50} = 30 μg/kg) or intraperitoneal (LD_{50} = 1.7 mg/kg) injection. The venom is also highly active in cockroaches (LD_{50} = 7 mg/kg).

2. Materials and methods

2.1. Toxin isolation and sequencing

The ants were collected in the Amazonian rain forest (Department of Ucauali, Peru). Ants were held by pincers on a sponge and venom obtained by tactile stimulation of the abdomen. The crude venom was extracted from the sponge and lyophilized. 40 mg of crude venom of *E. tuberculatum* ant was dissolved in 1 ml 0.1 M NH_4HCO_3 buffer (pH 8.1) and was applied to a Sephadex G-50F column (2.5 × 90 cm) in the same buffer. The toxic fraction was further purified by HPLC using a reverse-phase Ultrapore RPMC column (0.46 × 25 cm) Beckman in a linear gradient of acetonitrile in 0.1% trifluoroacetic acid. Before sequencing of Ea disulfide bonds were reduced with dithiothreitol and SH groups were pyridylethylated with vinylpyridine. The A and B chains of Ea were separated by reverse-phase chromatography. Each chain was digested with *Staphylococcus* endoproteinase V8, trypsin or endoproteinase Arg-C. All overlapping peptide fragments were sequenced on a gas-phase automatic sequenator 470A (Applied Biosystems, USA) leading to the complete amino acid sequences of the A and B chains.

2.2. NMR measurements and structure calculation

NMR measurements were performed on a Varian Unity 600 spectrometer using 2.5 mg Ea in 150 μl aqueous solution, pH 3.0. Resonance assignments were obtained from NOESY (mixing times 100 and 200 ms), TOCSY (50 ms) and double quantum-filtered COSY spectra recorded in H_2O and D_2O solutions at 10 and 30°C, following standard procedures [4].

For NMR structure determination, cross-peaks in NOESY spectra of Ea at 10°C were quantified using the EASY program [5] and interproton distance constraints were generated by the MARDIGRAS program [6]. After preliminary structure calculations with the distance geometry program DIANA [7] a new set of constraints was obtained: (i) assignments of more cross-peaks were found and some cross-peaks were re-assigned; (ii) MARDIGRAS-derived distance constraints were made more precise; (iii) hydrogen bonds between slowly exchanging D_2O amide groups and identified carbonyl groups were implied; (iv) three disulfide bridges were identified and appropriate distance constraints were used; (v) stereo-specific assignments were obtained by the GLOMSA program [7] for methylene and isopropyl groups and NOE-derived distance constraints were set accordingly. The final set of structures was calculated with the DIANA program with constraints on 755 NOE distances (404 intra-residue, 178 sequential, 92 medium-range, 29 long-range and 52 inter-chains), 33 hydrogen bonds, 3 disulfide bonds and 215 dihedral angles (64 φ, 68 ψ, 54 χ^1, 23 χ^2, 4 χ^3 and 2 χ^4 angles.

2.3. Conductance measurements

A planar lipid membrane was formed from monolayers of phosphatidylcholine in *n*-hexane (1 mg/ml) by the tip-dip method [8]. *Trans* and *cis* compartments contained (in mM) 145 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, pH 7.4, for symmetrical buffer solutions, and 145/48 (*cis*/*trans*) NaCl and other additives as above for unsymmetrical buffer solutions. The mean number of Ea monomers in a pore complex (N) was determined from analysis of the concentration (C) and voltage (V) dependence of steady-state conductivity (G) as described [9]: $G = \Gamma \cdot C^N \cdot \exp(V/V_e)$, where Γ and V_e are constants.

*Corresponding author. Fax: (7) (095) 330 7103.

Abbreviations: Ea, ectatomin; 2D, two-dimensional; COSY, 2D homonuclear correlated spectroscopy; NOESY, 2 D homonuclear NOE spectroscopy; TOCSY, 2 D homonuclear total correlation spectroscopy; RMSD, root mean square deviation.

SSDI 0014-5793(94)00518-Z

3. Results and discussion

The major toxic principle of the venom was isolated by a combination of gel-filtration and HPLC techniques and its amino acid sequence was determined (Fig. 1a). The toxin is a protein of molecular weight 7,928 Da composed of two polypeptide chains held together by disulfide bonds. The protein was named ectatomin (Ea), bearing in mind the species from which it was isolated and the abundance of amino groups within the molecule (calculated pI = 9.95). Ea accounts for 15–18% of crude venom and has an LD_{50} = 6.8 μg/kg at intracerebroven-

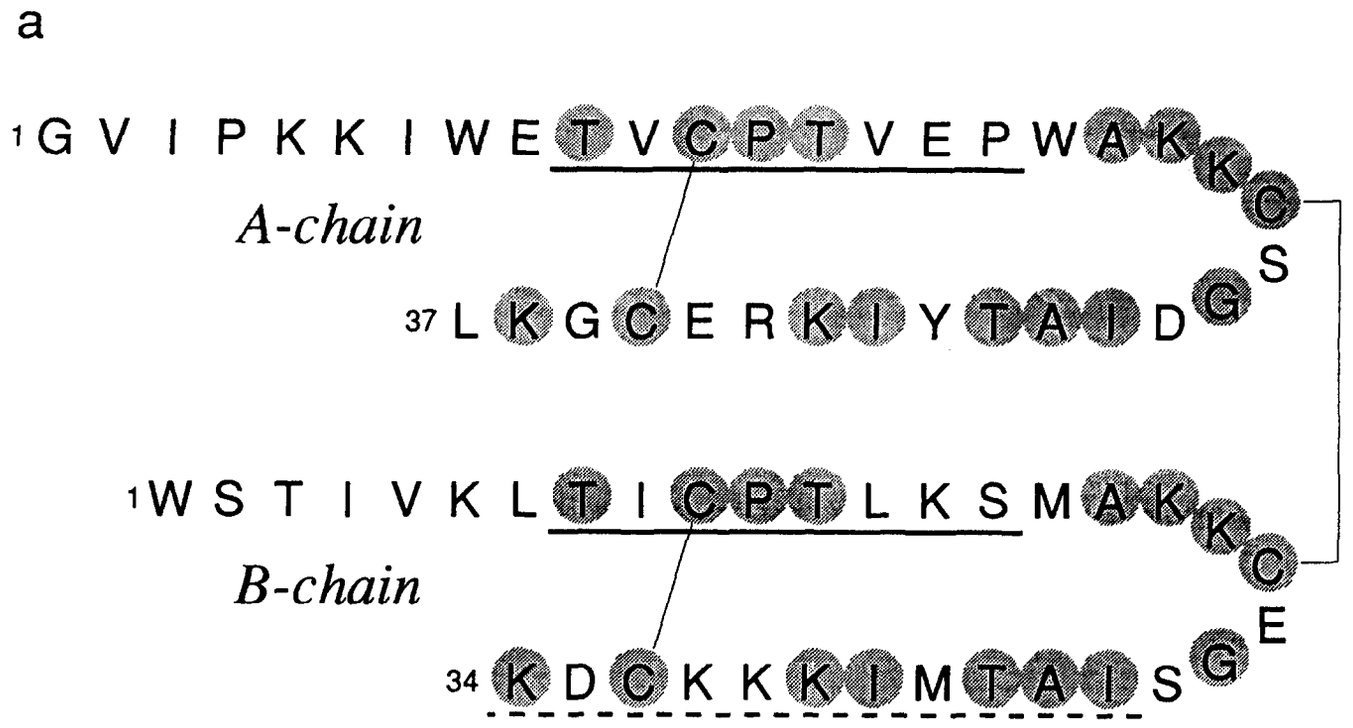

Fig. 1. (a) Sequence (single letter code) of two chains of ectatomin. The location of the disulfide bridges was determined from NMR data. Homologous amino acids in both chains are denoted within gray circles. Sequence homologies with the conservative site of kinase-related transforming proteins and the interferon γ-induced protein are underlined by solid and dashed lines, respectively. (b) Superposition of 20 NMR conformations (only backbone heavy atoms and heavy atoms of cysteine residues are shown in stereo view) of Ea in aqueous solution.

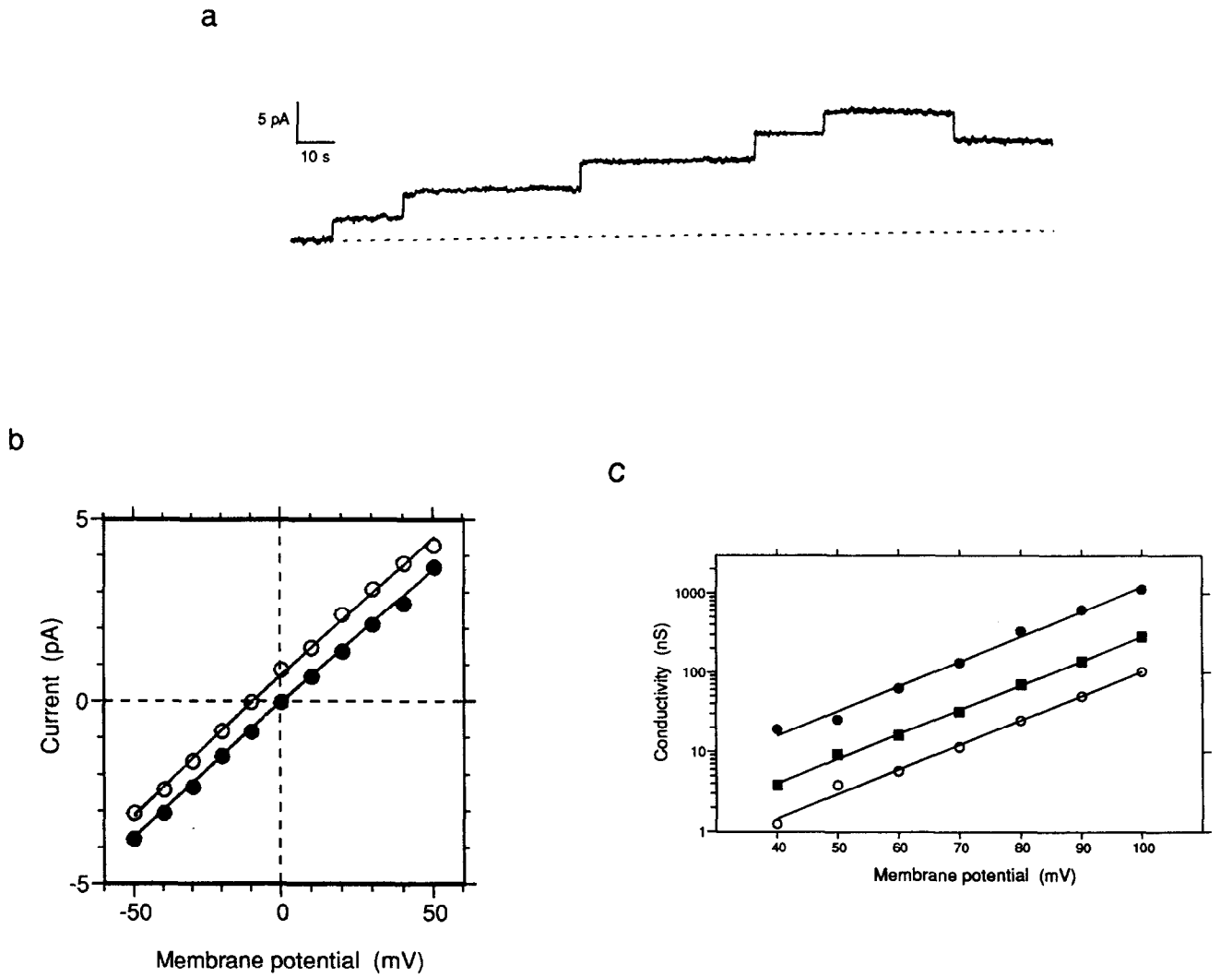

Fig. 2. (a) Current transitions for a bilayer membrane in the presence of 10^{-8} M Ea (*cis*-side only). (b) Current–voltage relationships in the presence of 10^{-8} M Ea (*cis*-side only). ●, symmetrical bath/pipette salt solutions; ○, unsymmetrical bath/pipette salt solutions (see section 2). (c) Voltage dependence of steady-state Ea-induced conductivity. Experimental data at $3 \cdot 10^{-8}$ M (○), $5 \cdot 10^{-8}$ M (■), 10^{-7} M (●) Ea in the *cis* aqueous phase compartment and lines theoretically predicted for the same Ea concentrations with constants $\Gamma = 9 \cdot 10^4$ S·M^{-2}, $V_e = 14$ mV and $N = 2$ (see section 2).

tricular injection in mice. Its toxic effect in cockroaches (LD_{50} = 2.1 mg/kg) was similar to that observed with the crude venom. Thus Ea accounts for ≅ 90% of total toxicity of the crude venom.

In order to obtain more insight into the structure–function relationship of Ea we studied its spatial structure by means of NMR spectroscopy. A set of 20 conformations with DIANA target function values of 4.89 ± 0.34 Å^2, maximal violations 0.4 Å for distances, 0.2 Å for van der Waals constraints, and 6° for dihedral angles, was obtained (Fig. 1b). The RMSD after superimposing any of two structures are 1.2 ± 0.3 Å for all backbone atoms and 1.7 ± 0.3 Å for all heavy atoms. If residues 5–34 of chain A and 3–32 of chain B were considered the RMSD was 0.6 ± 0.2 Å for backbone atoms and 1.3 ± 0.2 Å for all heavy atoms.

Ea in aqueous solution forms a bundle of four α-helices (Fig. 1b). The spatial structure of the two Ea chains are similar (Fig. 1b), which accords with their rather high sequence homology (Fig. 1a). Each chain consists of two antiparallel α-helices connected by the hinge region of 4 residues and a disulfide bridge. The two hinge regions are further connected by a third disulfide bridge. The four helices are amphipathic, and in the aqueous solution, non-polar side chains form a hydrophobic interior and polar groups protrude from the molecule into the surrounding milieu.

A comparison of amino acid sequences of the A and B chains of Ea (Fig. 1a) with the known sequences in the protein–peptide bank PIR revealed no distinct homology with other toxins. However, sequences 10–17 of chain A and 8–15 of chain B of Ea (Fig. 1a) are highly homologous with the conservative site (residues 253–260: TVCPTVKP) of tyrosine kinase-related transforming proteins from different sources [10,11]. Another marked homology is that of the B-chain sequence 24–34 with

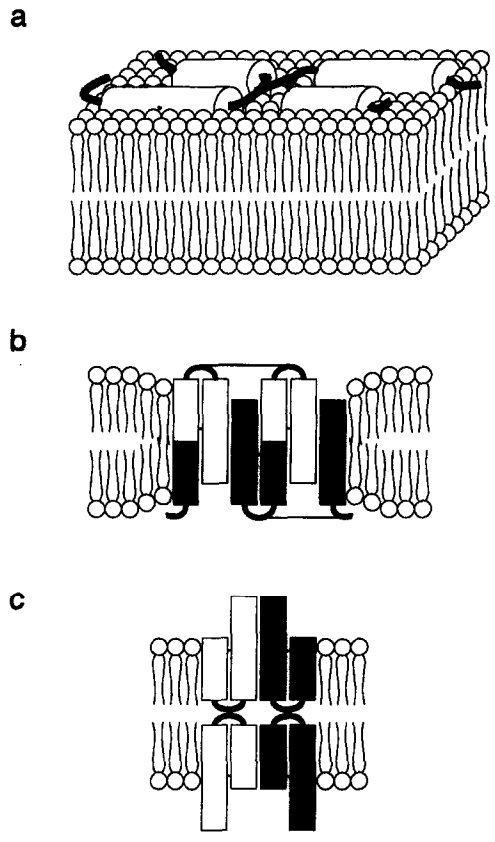

Fig. 3. Scheme for protein insertion into membranes suggested by the structure of Ea in aqueous solution. (a) Conformational reorganization of the Ea molecule, so that hydrophobic surfaces of amphipathic helices are submerged into the lipophilic part of the membrane. (b and c) Two possible models of ectatomin dimerization and pore formation.

residues 63–72 (IATMKKKCEK) of interferon γ-induced protein [12].

The sequence homologies found might suggest an intracellular mode of Ea functioning which requires its translocation through the cell membrane. With this hypothesis in mind we studied the interaction of Ea with bilayer membranes. Experiments with phosphatidylcholine bilayers showed (Fig. 2a,b) that Ea gives rise to pores weakly selective for cations over anions ($P_{Na^+}/P_{Cl^-} = 2$) with a conductance level of 70 pS. The pore formation is voltage dependent. Pore activation occurs primarily at *cis*-positive potentials (Fig. 2c), when the local concentration of a positively charged Ea molecule (+ 9 e.u. at physiological pH's) increases in the proximity of the membrane. The concentration dependence of pore formation suggests that 2.0 ± 0.2 Ea molecules are needed to form a pore (Fig. 2c).

Thus, Ea can enter membranes and form pores. Taking into account that (i) the Ea pore in bilayer membranes is a dimer and channel conductance is identical in both directions (Fig. 2b), (ii) secondary structures of Ea in aqueous solution and in the membrane-mimicking solvent, methanol, according to CD measurements (data not shown), (iii) the α-helical hairpin of each chain A or B is fixed by disulfide bridges, and (iv) the length of Ea helices (15–25 Å) is too short to cross an undistorted bilayer ($\geq$30 Å) the following schemes of Ea pore formation were proposed (Fig. 3). A positively charged Ea molecule ensures a high local concentration of Ea close to the membrane and increases the non-specific membrane affinity. The membrane destroys the electrostatic and hydrophobic interactions between the A and B chains, two α-helical hairpins come apart and stick to the membrane so that the hydrophobic surfaces of amphipathic helices are submerged in the lipophilic part of the membrane (Fig. 3a). In the presence of a transmembrane potential the helical axes tend to orientate across the bilayer and two Ea molecules aggregate by the polar faces of the helices, the lipophilic surfaces facing the membrane interior. Several models of pore structure are possible. For instance, the bilayer can be locally distorted so as to accommodate the length of mis-match (Fig. 3b). The alternative is that the pore is formed by deployed Ea molecules (Fig. 3c). The scheme has to be further tested against experimental data.

Thus the observations are consistent with the inside-out mechanism of conformational rearrangement of the Ea molecule. The inside-out mechanism may be a general principle for insertion and membrane translocation of water-soluble proteins. Such a mechanism has been considered for the membrane-attack complex of the complement cascade [13], for insertion of membrane protein precursors [14], bacterial protein toxins [15] and others. All bacterial protein toxins (colicins, insecticidal δ-endotoxin, exotoxin A and diphteria toxin) after crystallization from water contain a bundle of α-helices and form ion pores in artificial membranes similar to Ea. However, in contrast to bacterial toxins which are large proteins (molecular weight $\geq$60 kDa) Ea is a small protein. This opens up a unique chance for the detailed study of the mechanism of insertion of water-soluble proteins into biological membranes by current methods of structural biology.

The pore formation in cell membranes might explain the toxic activity of the Ea molecule. However, taking into account the homologies mentioned above (Fig. 1a) we cannot exclude the possibility of Ea translocation across membranes and subsequent intracellular functioning.

Acknowledgements: We thank A.A. Zacharov for help with identification of ant species and assistance in collection of the ants, and V.T. Ivanov and Yu.A. Chizmadzhev for discussions and comments on the manuscript.

References

[1] Blum, M.S. (1992) J. Toxicol. 11, 115–164.
[2] Schmidt, J.O., Blum, M.S. and Overal, W.L. (1986) Toxicon 24, 907–921.

[3] Schmidt, J.O. (1986) in: Venoms of Hymenoptera (Piek, T., ed.) pp. 425–508, Academic Press, New York.
[4] Wuthrich, K., Wieder, G., Wagner, G. and Braun, W. (1982) J. Mol. Biol. 15, 311–319.
[5] Eccles, C., Guntert, P., Billiter, M. and Wuthrich, K. (1991) J. Biomol. NMR 1, 111–130.
[6] Borgias, B.A. and James, T.L. (1990) J. Magn. Reson. 87, 475–487.
[7] Guntert, P. and Wuthrich, K. (1991) J. Biomol. NMR 1, 447–456.
[8] Coronado, R. and Latorre, R. (1983) Biophys. J. 43, 231–236.
[9] Latorre, R. and Alvarez, O. (1981) Physiol. Rev. 61, 77–150.
[10] Sukegawa, J., Semba, K., Yamanashi, Y., Nishizawa, M., Miyajima, N., Yamomoto, T., Toyoshima, K. Mol. (1987) Cell. Biol. 7, 41–47.
[11] Kitamura, N., Kitamura, A., Toyoshima, K., Hirauma, Y., Yoshida, M. (1982) Nature 297, 205–208.
[12] Luster, A.D., Unkeless, J.C. and Ravetch, J.V. (1985) Nature 315, 672–676.
[13] Bhakdi, S. and Tranum-Jensen, J. (1987) J. Rev. Physiol. Biochem. Pharmac. 107, 148–166.
[14] Wickner, W. (1988) Biochemistry 27, 1081–1086.
[15] Parker, M.W. and Pattus, F. (1993) Trends Biochem. Sci. 18, 391–395.