Biochemical and Biophysical Research Communications 427 (2012) 385–391

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

A high resolution electro-optical approach for investigating transition of soluble proteins to integral membrane proteins probed by colicin A

Alf Honigmann [a,1], Lakshmi Padmavathi Pulagam [b,2], Michael Sippach [a,b], Philipp Bartsch [a], Heinz-Jürgen Steinhoff [b], Richard Wagner [a,*]

[a] Universität Osnabrück, FB Biologie/Chemie, Barbara Str. 13, 49076 Osnabrück, Germany
[b] Universität Osnabrück, FB Physik, Barbara Str. 7, 49076 Osnabrück, Germany

ARTICLE INFO

Article history:
Received 6 September 2012
Available online 18 September 2012

Keywords:
Membrane
Artificial bilayer
Fluorescence
Electro-optical recording
Colicin A
Colicin A-oligomerization
Membran-Protein insertion

ABSTRACT

The transition from water soluble state to an integral membrane protein state is a crucial step in the formation of the active form of many pore-forming or receptor proteins. Albeit this, high resolution techniques which allow assay of protein membrane binding and concomitant development of the final active form in the membrane await further development. Here, we describe a horizontal artificial bilayers setup allowing for simultaneous electrical and optical measurements at a single molecule level. We use the membrane binding and subsequent channel formation of colicin A (ColA) a water soluble bacteriocin secreted by some strains of *Escherichia coli* to demonstrate the potential of the combined electro-optical technique. Our results expand the knowledge on ColA molecular details which show that active ColA is monomeric; membrane binding is pH but not membrane-potential ($\Delta\phi$) dependent. ColA is at $\Delta\phi$=0 permeable for molecules $\geqslant$1 nm. Although ColA exhibits low ion conductance it facilitates permeation of large molecules. Our electro-optical recordings reveal ColA monomeric state and the chimeric character of its pore.

1. Introduction

Pore-forming toxins are secreted by many pathological bacteria as a weapon against host cells or against competing bacteria [1,2]. The mechanism of action generally involves the secretion of the monomeric toxin into a water soluble conformation. Upon contact of the toxin with the target cell-membrane a conformational change is triggered which induces membrane insertion and in most cases an oligomerization process. The toxic action of colicins is either nuclease activity in the cytosol or ion-channel formation in the plasma membrane of the target *Escherichia coli* cells. The crystal structures of the water-soluble conformation revealed that all pore-forming colicins are arranged in three domains [3,4]. The domains were named according to their specific function: the receptor binding domain, the translocation domain and the channel-forming domain [5,6]. At the plasma membrane the pore-forming domain inserts into the bilayer and forms a voltage-dependent ion channel which short circuits the vital electro-chemical gradient of the target cell [7,8]. The high resolution structure of the water soluble conformation of the pore-forming domain of ColA shows a ten-helix bundle containing a central hydrophobic helical hairpin in solution [3]. The structural similarities between colicins and other pore forming toxins suggest that a conserved mechanism of pore-formation is at work.

Contradicting properties of the open channel have greatly complicated the establishment of an appropriate structural model: even though the ion conductivity of the open conformation is very low, but nevertheless large ions or even small folded proteins can be translocated by ColE1 and Col-Ia [9–11]. Different studies point to a monomeric pore [6,12,13], but also to oligomeric pores of Col IA in lipid bilayers [14].

Here we used an electro-optical approach to characterize the membrane binding of ColA and the subsequent channel opening upon application of an electrical field. Our electro-optical setup is comprised of a horizontal lipid bilayer chip, representing a modified version of the planar lipid bilayer technique and a 3D-confocal laser scanning microscope equipped with single photon detection unit. This setup allows single channel conductance- (voltage clamp), diffusion- (FCS), environment- (fluorescence lifetime) and oligomerization- (FRET, FIDA) measurements, simultaneously. Accessing these parameters simultaneously is promising in the context of ColA because to study incorporation into the membrane

* Corresponding author. Address: Biophysics, Dep. Biology/Chemistry, University Osnabrueck, Barbarastr. 13, 49076 Osnabrueck, Germany. Fax: +49 541 969 2243.
E-mail address: wagner@uos.de (R. Wagner).
[1] Present address: Max-Planck-Institut für Biophysikalische Chemie, Abteilung NanoBiophotonik, Am Faßberg 11, 37077 Göttingen, Germany.
[2] Present address: Department of Epidemiology and Biostatistics, Case Western Reserve University, Cleveland, USA.

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http://dx.doi.org/10.1016/j.bbrc.2012.09.069

and the open channel conformation it is required to have a controllable and stable membrane potential which was lacking in previous studies on colicins.

2. Materials and methods

2.1. Chemicals

E. coli polar lipid extract was purchased from Avanti Polar Lipids (Alabaster, AL). *N*,*N*-dimethyl-*N*-(iodoacetyl)-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl) ethylenediame (NBD amide) was purchased from Molecular Probes (Eugene, OR). The organic dyes Atto488-maleimide and Atto647N-maleimide were purchased from Atto-Tec (Siegen, Germany). Lipids were stored in methanol/chloroform (1:1) under nitrogen at −20 °C.

2.2. Mutagenesis, preparation and purification of ColA

The plasmid pLR1, which encodes the wild-type ColA, was used as template to replace different residues within the ColA sequence with a cystein. Mutagenesis, activity test of mutant strains and preparation of the different Cys-mutant proteins was performed essentially as described [15].

2.3. Fluorescence labeling of single cysteine ColA mutants

ColA-NBD and $ColA_x$-488 and $ColA_x$-647N were produced as described [15]. The products ColA-F were separated from non-reacted dyes by size exclusion chromatography using NAP-5 columns (GE Healthcare, Piscataway, NJ).

The degree of labeling (DOL) was determined by absorption spectroscopy. Protein concentration was estimated from A_{280} (corrected by the dye absorption at 280 nm) with a calculated ε of 51,910 M^{-1} cm^{-1} (Expasy ProtParam). The resulted DOLs varied between 0.6 and 0.9.

2.4. Horizontal lipid bilayers and electrophysiology

The construction and handling of the horizontal bilayer chip (HLB) was as described in detail [16]. Bilayer formation was monitored optically and electrically. Fluorescence fluctuation analysis and electrical measurements were performed essentially as described [16].

2.5. Calcein efflux from liposomes incubated with ColA

Calcein efflux from liposomes incubated with ColA was monitored as described in the Supplements.

2.6. Confocal microscopy setup

Confocal imaging and fluorescence fluctuation recordings and analysis were performed on a modified Insight Cell 3D microscope from Evotec technologies (Hamburg, Germany, now Perkin Elmer), as described in detail [16].

2.7. Steady state fluorescence measurements

Steady state fluorescence measurements were performed with a Jasco FP 6500 fluorimeter (Jasco, Gross-Umstadt, Germany) as describe in Supplemental Fig. S2.

3. Results

3.1. Membrane binding of ColA is pH dependent

We studied the *in vitro* binding of ColA to membranes made of natural *E. coli* lipids using horizontal lipid bilayers as well as large uni-lamellar vesicles (LUV). ColA was labeled either with the fluorescent dye NBD to probe a change in hydrophobicity upon membrane binding or with Atto488, 647 to measure the diffusion properties in aqueous solution and at the bilayer. Fig. 1A shows a typical bilayer from *E. coli* lipids, the membrane was imaged by scanning the XZ plane. Without fluorescent label the bilayer is not visible on the image (Fig. 1A and B). Only the auto-fluorescence of the PTFE foil and the lipid torus are observable. When ColA166-488 88 was added to the trans compartment of the bilayer chip ($C \approx 10$ nM) the fluorescence of ColA166-488 88 is clearly visible on the trans side of the bilayer. The bilayer is indirectly visible, since it acts as a diffusion barrier for ColA166-488 88. Binding to the bilayer was studied at pH 7 (Fig. 1A) and pH 4 (Fig. 1B), respectively. At pH 7 no significant accumulation of $ColA_{166}$-488 at the bilayer was detectable (Fig. 1A), while at pH 4 binding to the bilayer was detected directly after addition of $ColA_{166}$-488 (Fig. 1B). The bright spots in the trans solution indicate that the protein in solution tended to aggregate at low pH (Fig. 1B).

A holding potential of +100 mV (positive on the side of ColA addition) did not significantly increase ColA166-488 88 binding (data not shown). We therefore can conclude that an applied membrane potential does not affect membrane binding of ColA.

3.2. Electrophysiological properties of single ColA channels

The concentration of ColA added to the bilayer was adjusted to result either in single or multi-channel activities that were dependent on pH (see Figs. 1 and 2). For the analysis of the single channel conductance of ColA a trans-membrane potential of 80–100 mV was applied to induce the open channel conformation. The typical single channel current trace at pH 7 (Fig. 2A) reveals gating of the ColA channel into several conductance states. The mean single channel conductance at different membrane potentials (Fig. 3B) revealed conductance histograms with peak values of $G_{max} = 10ps$ at pH 7 and $G_{max} = 10ps$ at pH 4, (1 M KCl) respectively. Interestingly, the mean single channel conductance at pH 4 was significantly lower (≈ 2-fold) compared to pH 7,which could be an indication for a reduced pore diameter and/or changes in the electrostatics of the channel pore due to protonation of amino acid residues at pH 4. As observed for other members of the colicin family [9] the single channel conductance of ColA is even lower than the one of the narrow gramicidin A channel [17].Using the approximation of a water filled cylindrical pore [19] with a restriction zone of 2 nm, the low conductance would imply a diameter of $d_{ch} = 1.6$ Å of the ColA channel (see Supplement). The strength of membrane potential necessary to open the channel (see Fig. 2D) displays a steep exponential dependence on the membrane potential with $v_p = 0.5 \approx 90mv$. Hence, the membrane potential values for efficient open channel formation (+90 mV) determined *in vitro* would be sufficient for channel incorporation with $0.5 < p_{open} < 1$ in the *E.coli* plasma membrane *in vivo* with reported values of $V_m \cong +85$ mV, pH 5 and $V_m \cong +145$ mV, pH 8, as well [18].

The dependence of open? channel formation on the electrical field was tested with an ensemble of channels incorporated in the bilayer. A trans-membrane potential of 80 mV was applied for 15 s, which resulted in a linear increase of current due to steady/successive? opening of ColA channels (Fig. 2C). When the direction of the applied potential was abruptly inverted, the number of open channels decreased exponentially with a time constants of