Electrophysiological Behavior of the TolC Channel-Tunnel in Planar Lipid Bilayers

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Abstract. Escherichia coli TolC assembles into the unique channel-tunnel structure spanning the outer membrane and periplasmic space. The structure is constricted only at the periplasmic entrance of the tunnel and this must be opened to allow export of substrates bound by cognate inner membrane complexes. We have investigated the electrophysiological behavior of TolC reconstituted into planar lipid bilayers, in particular the influence of the membrane potential, the electrolyte concentration and pH. TolC inserted in one orientation into the membrane. The resultant pores were stable and showed no voltage-dependent opening or closing. Nevertheless, TolC could adopt up to three conductance states. The pores were cation-selective with a permeability ratio of potassium to chloride ions of 16.5. The single-channel conductance was higher when the protein was inserted from the side with negative potential. It showed a nonlinear dependence on the concentration of the electrolyte in the bulk solution and decreased as the pH was lowered. The calculated pK of the apparent closing was 4.5. The electrophysiological characterization is discussed in relation to the TolC structure, in particular the periplasmic entrance.

Key words: TolC — Electrophysiology — Channel-tunnel — Type I secretion — Multidrug efflux pump

Introduction

TolC is a trimeric outer membrane protein of Escherichia coli. It is central to the type I export of large protein toxins (Koronakis, Koronakis & Stauffer, 1997; Wandersman & Delepelaire, 1990) and to multidrug efflux (Fralick, 1996; Zgurskaya & Nikaido, 2000). During export, it is recruited by specific substrate-bound, energized complexes in the cytoplasmic membrane. In this way, substrates bypass the periplasm and exit the cell (Koronakis et al., 1997; Thanabal et al., 1998). TolC is anchored in the outer membrane by a 40-Å long β-barrel (the channel domain) and spans the periplasmic space via a contiguous 100-Å long α-helical barrel (the tunnel domain). The assembled channel-tunnel forms a single pore with a diameter of 35 Å, providing a 43,000 Å² water-filled exit duct (Koronakis et al., 2000). While the TolC outer membrane channel is constitutively open to the cell exterior, towards the tunnel periplasmic entrance the inner diameter decreases to a virtual close to provide the only constriction of the structure. The entrance must therefore be opened and it is envisaged that the substrate-dependent recruitment of TolC triggers an iris-like opening involving movement of the three pairs of α-helices that form the aperture (Koronakis et al., 2000). The principal structural elements of TolC are conserved throughout a large family of homologues, suggesting that this is a common mechanism underlying export and efflux events throughout Gram-negative bacteria (Koronakis et al., 2000; Andersen, Hughes & Koronakis, 2000).

Channel-tunnel behavior can now be investigated in the context of the structure, which is distinct from other outer membrane proteins (Cowan et al., 1992; Forst et al., 1998; Andersen et al., 2000). This study presents an electrophysiological characterization of purified TolC reconstituted into lipid bilayers. While some of the experimental conditions applied are unphysiological, they facilitate understanding of the electrophysiological behaviour of this protein and establish a basis for a study of the TolC structure function relationship.
Materials and Methods

TolC Protein Purification

TolC protein was expressed and purified after isopropyl β-D-thiogalactopyranoside (IPTG)-induction of E. coli BL21 (DE3) carrying the recombinant plasmid pT7TolC (Koronakis et al., 2000). Cells were broken in a French press, the membranes collected (50,000 × g, 40 min) and washed twice in 20 mM Tris HCl, pH 7.4, 20 mM MgCl₂, 0.5% Triton X-100 (Bio-Rad). TolC was solubilized from the membrane in 20 mM Tris- HCl, pH 7.4, 20 mM MgCl₂, 5% Triton X-100, 10% glycerol, and insoluble material removed (50,000 × g, 20 min). TolC was then purified from the supernatant by binding on a Q sepharose anion exchange column (5ml Hitrap Q, Pharmacia) and elution with a NaCl gradient in 20 mM Tris, pH 7.4, 0.5% Triton X-100. The TolC was pure as analyzed by SDS-PAGE.

Conductance Measurements

Instrumentation comprised a Teflon chamber with two aqueous compartments connected by a small circular hole (diameter 0.5 mm). Black lipid membranes were formed as described previously (Benz et al., 1978), by painting onto the hole a 1% solution of diphytanoyl phosphatidylcholine (Avanti Polar Lipids, Alabaster, AL) in n-decane. The aqueous KCl solutions (Aldrich) were buffered by HEPES and citrate (Sigma, Poole, Dorset, UK). The temperature was kept at 20°C throughout. TolC was diluted in 0.5% Triton and was added to one side of the membrane (there were no differences when protein was added to the front or the back compartment of the apparatus, excluding asymmetries in the experimental setup). The membrane current was measured with a pair of calomel electrodes connected in series to a voltage source and an electrometer (Keithley 6517A, Reading, UK). The resistance of each membrane under the specific conditions was tested before adding TolC to the aqueous solution. Only membranes with resistance higher than 200 GΩ measured at a potential of 120 mV were used to perform the experiments. Control experiments confirmed that neither electrolyte concentration, pH, nor high potential as used in our measurements had an effect on the membranes. For single-channel recordings the electrometer was replaced by a current amplifier (Keithley 426). The amplified, filtered signal (filter rise time 30 msec) was monitored using a strip chart recorder. It was recorded with a sampling rate of 50 sec⁻¹ by a PC connected to the output signal by an A/D-converting card (Keithley DAS-1601).

Selectivity Measurements

For the zero-current membrane potentials the membranes were formed in a 30 mM KCl solution (1 mM HEPES, 7.5). TolC was added to either side of the membrane and the increase of the membrane conductance due to insertion of pores was observed with the electrometer. When a conductance of at least 2 nS was reached, corresponding to at least 100 inserted TolC channels (the single-channel conductance of TolC in 30 mM KCl is around 20 pS), the instrumentation was switched to the measurement of the zero-current potential and the KCl gradient was established by adding 3 mM KCl solution to one side of the membrane while stirring. The zero-current membrane voltage reached its final value after 2–5 min. Single-channel experiments were used throughout to provide information on the changes in conductivity, noise and substate-switching frequency, but the results were confirmed in specific multichannel experiments.

Results

Influence of the Membrane Potential on TolC Conductance

The TolC structure is highly asymmetric (Fig. 1), dictating channel insertion into the bacterial outer membrane with the tunnel domain projecting into the periplasmic space. We first assessed the dependence of the single-channel conductance of membrane-inserted TolC on the polarity of the applied potential. TolC was added to one side of black lipid membranes. Throughout, this side is referred to as the TolC-proximal side, and the potential is always given with respect to this side. The conductance was measured at either +80 mV (Fig. 2 upper trace) or −80 mV (Fig. 2 lower trace). After addition of TolC protein, conductance increased step-wise as single TolC molecules inserted into the artificial bilayer. Analysis of at least 100 single insertion events showed that the polarity of the membrane potential affected the conductance of TolC. The mean conductance was 81.5 ± 1.0 pS when the potential was positive, and 89.2 ± 1.7 pS when it was negative. It was evident that TolC can adopt or ‘switch into’ more than one conductance substate (Fig. 2 inset). When the potential was positive, insertion did not increase the noise (when compared to the zero line), but when the potential was negative the noise increased with each newly inserted TolC.

To confirm that the polarity-dependent behavior was an intrinsic property of membrane-inserted TolC, we assayed the influence of membrane potential in a single-channel experiment. In this case, only