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Generation of giant protoplasts of *Escherichia coli* and an inner-membrane anion selective conductance

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Abstract

Established methods were modified and combined to generate unilamellar giant protoplasts in order to study the electric events on the cytoplasmic membrane of *Escherichia coli* with patch-clamp technique. The activities of many types of conductances were encountered, one of them is characterized here. This channel conductance is 109 pS in 150 mM KCl; it prefers anions, it is highly voltage dependent and is blocked by micromolar concentrations of anthracene-9-carboxylic acid.

Keywords: Protoplast; Inner membrane; Patch clamp; Anionic channel; (*E. coli*)

1. Introduction

Electrophysiological studies on *Escherichia coli* provide a unique opportunity because of the wealth of genetic, physiological and molecular-biological knowledge already accumulated in this species. With the advent of the patch-clamp technique [1] electrical measurements can be done not only on giant bacterial cells in vivo, or by incorporation of bacterial ion channels onto planar lipid bilayers in vitro, but as this study shows it is possible to measure minute currents carried by ions through single channel proteins in the native cytoplasmic membrane.

E. coli, like other Gram-negative bacteria, has two membranes separated by periplasm and the peptidylglycan cell wall. The wall and the two membranes are collectively referred to as the cell envelope. The outer membrane contains 14 identified proteins [2]. This membrane plays mostly protective role in bacterial physiology. The inner membrane is the cytoplasmic membrane, which houses the machinery for electron transport, nutrient uptake, chemoreception, etc. With live *E. coli* preparation, only the outer membrane of the bacterium giant spheroplasts (two membrane objects) was investigated with the patch-clamp technique [3,4]. These studies have revealed the activity of two mechanosensitive channels and have modified the common understanding of the outer membrane being a molecular sieve [5–7].

Given the theoretical and practical importance of *E. coli*, a better understanding of its cytoplasmic membrane in ionic and electric terms seems valuable. This paper describes a method to study the inner membrane of *E. coli* giant protoplasts (one-membrane objects, containing only cytoplasmic membrane) with the patch-clamp electrodes. From many channels encountered an anion selective channel has been selected for this presentation because of many common features it shares with anion selective channels from other cells.

2. Materials and methods

2.1. Bacterial strains

Frag5 (F^-, kdpABC5, *thi*, *rha*, *lacZ*, derived from *E. coli* K12) and Frag127 (Frag5 with *kefC*::Tn*10*) were kindly provided by Wolfgang Epstein (University of Chicago, USA). Data presented were obtained from Frag127. Similar results were obtained with Frag5. The genotypes of these strains were unrelated to the channel under investigation here.

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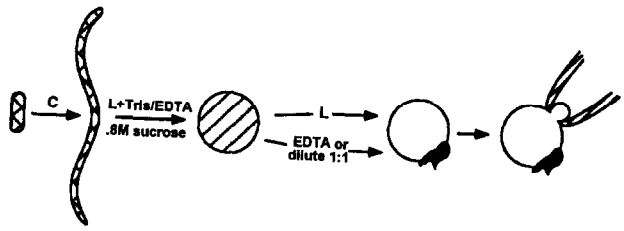

Fig. 1. Preparation of giant spheroplasts (dashed circle) and giant protoplasts (open circles) for patch-clamp studies. See text for explanation.

2.2. Preparation of giant spheroplasts

The method was largely as described previously [3]. Briefly, bacteria were cultured overnight in modified Luria-Bertani medium having NaCl substituted with KCl, collected and transferred to the same medium with cephalexin at 60 μg/ml ('C' in Fig. 1). In the presence of cephalexin that blocks septation, *E. coli* grew into long filaments. These filaments were harvested and the pellet was resuspended in 2.5 ml of 0.8 M sucrose ('Suc') and 10 mM Tris-HCl (pH 8.0). Addition of 120 μl of lysozyme (5 mg/ml) ('L') and 6 mM EDTA (pH 8.0) gradually converted them into giant spheroplasts 3 to 10 μm in diameter [8,9]. These giant spheroplasts could be stored at −20°C over months and retained channel activities upon thawing.

2.3. Patch-clamp recording

Standard methods of patch-clamp were used [1]. All experiments were conducted at room temperature using a List Medical EPC7 patch-clamp amplifier. Recording and data processing were as described previously [10].

The method of generating giant protoplasts has been reported in an abstract form elsewhere [11] and adopted by Cui et al. [12] to investigate mechanosensitive channels in the inner membrane of *E. coli*.

3. Results

3.1. Generating giant protoplasts

It has been found that the generation of giant spheroplasts [3] followed by a variant of the procedure known to remove the outer membrane of ordinary spheroplasts [13] yielded a preparation of giant protoplasts suitable for patch-clamp studies. A few microliters of thawed giant spheroplasts were placed directly into the 0.5 ml patch-clamp experimental chamber with 150 mM KCl, 400 mM sorbitol and 10 mM Tris, pH 7.2. After the spheroplasts settled to the bottom of the chamber, 20 μM lysozyme followed by an addition of 1 mM EDTA was applied. The shapes and the refractivity of the spheroplasts were then followed under a phase contrast. It was possible to follow the peeling of the outer membrane off to one side of the spheroplasts and eventually discern some gray and non-refractile spheres, identified as protoplasts by Birdsell and Cota-Robles [13] (Fig. 2). Swelling apparently also helped the protoplasts to emerge. A one-to-one dilution of the 400 mM sorbitol bath with sorbitol-free buffer, for example, was found to generate usable protoplasts even without the addition of EDTA. In preparation for seal formation, $MgCl_2$ was added to a final concentration of 10 to 20 mM. The patch-clamp pipet was filled with a solution slightly hypotonic to the bath. The pipet solution contained 300 mM sorbitol but otherwise was the same as the bath. A seal of 5 to 10 GOhm could be formed between the pipet tip and the protoplast surface, usually under slight suction. After seal formation, the membrane patch was excised from the protoplast by air exposure, and the chamber was perfused with experimental solutions without divalent cations. The seal was fragile and did not survive repeated perfusions of the chamber. It has been found that the addition of 3 to 10 mM glutathione to the bath increased the stability and the longevity of the seal. This concentration of glutathione is in a physiological range for bacteria and glutathione was always present in the bath in all experiments.

Upon transfer to culture nutrient the protoplasts resumed growth and division within hours.

3.2. Channel activities in protoplasts patches

In excised inside out patches of protoplasts membranes, the activities of several types of ion channels have been consistently observed, each around 100 pS in conductance or less [4]. These results are in contrast with the findings of extensive experimentation with the outer membrane of

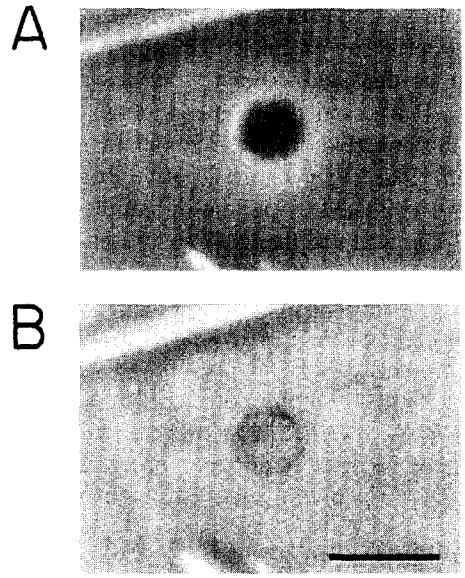

Fig. 2. An *Escherichia coli* giant spheroplast – a two membrane object (A) after treatment with lysozyme and EDTA becomes a unilammelar sphere – a giant protoplast (B). The bar represents 10 μm. The picture was taken under phase contrast microscope.

giant spheroplasts [3,6,14–17]. While patches of the spheroplast membrane, presumably the outer membrane, always revealed the activities of a mechanosensitive channel of approx. 1 nS conductance (MscS), its activities were not encountered in patches of from giant protoplasts. This drastic difference leaves little doubt that the surface of the putative giant protoplasts differs from that of the spheroplast in bioelectric terms. One of the several activities encountered, that of an anion channel is described here.

3.3. Single channel activities

This anion selective channel was encountered in approx. 10% of protoplast membrane patches. Results reported here summarize the experience from 37 patches that exhibited this channel activity. At lower positive voltages ($< +30$ mV), this channel showed bursting behavior. Interburst intervals were on the order of 1 s (Fig. 3A, top trace). Each burst consisted of repeated openings to at least three levels. The channel tended to dwell at the highest open level only at the beginning of the burst (middle trace). In most patches the bursting behavior of the channel disappeared with time so that the channel did not adopt three levels of conductance but several subconductances were observed instead (Fig. 3B) until it inactivated entirely. The channel displayed partial or complete inactivation within 1–10 min and the inactivation was more rapid the higher voltage was applied. The inactivation was irreversible and it could not be reversed by lower positive voltages. The channel was much more active upon positive pipet voltages than upon negative voltages (Fig. 3B, note the slow time base). The response to voltages seemed sluggish. Channel activities were not observed for several tens of seconds, when the membrane patch was returned to a positive voltage after being held at a negative voltage (data not shown). Channel activation also seemed to have a threshold. The threshold voltage varied from patch to patch. Fig. 3C showed two extreme cases when the threshold for channel activation was around +15 mV in one patch and around +45 mV in another one. The threshold voltage was always higher after the patch was being held at negative voltages.

3.4. Channel conductances

The bursting behavior and the activity structure within a burst suggest that the activities are from a single entity instead of a summation stochastic of activities of several channels. Fig. 3A showed that this channel could open to several conductance levels. Amplitude histogram of these activities indicated three peaks at around 1, 2 and 3 pA which correspond to 100, 200, and 300 pS, respectively. It was arbitrarily decided that the current level which was most frequently seen at lower positive voltages would be considered as a unit conductance. This conductance is marked by dashed line in Fig. 3A, bottom trace. The

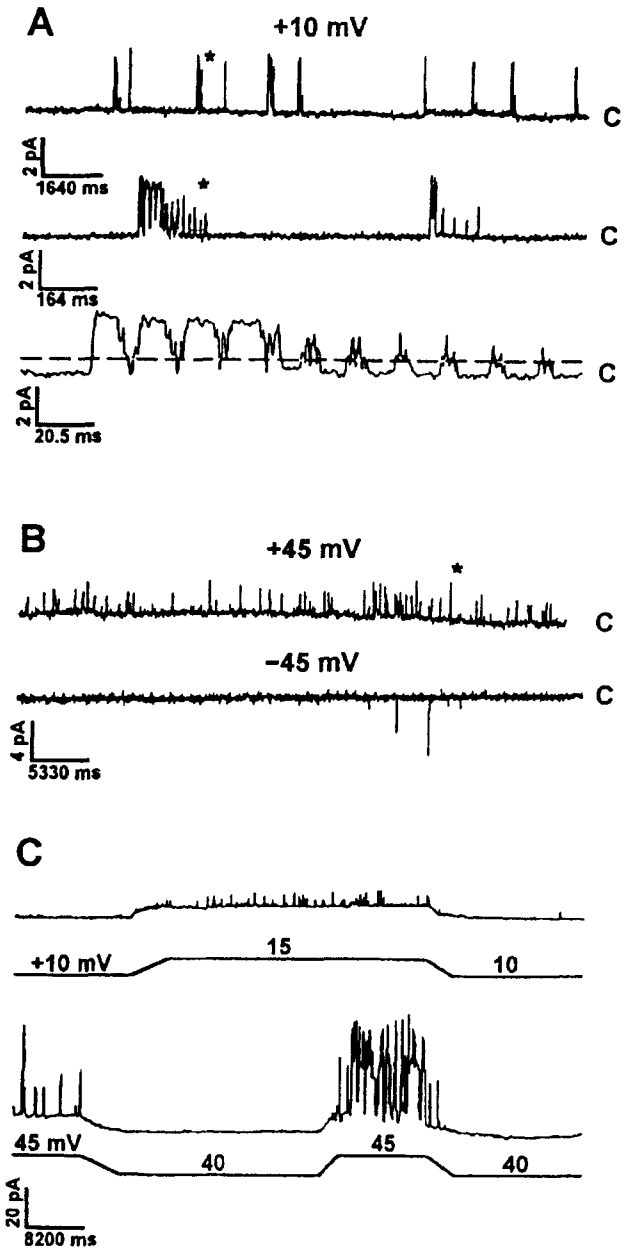

Fig. 3. Single channel activities of the anion selective channel from the *E. coli* inner membrane. (A) The bursting behavior of the channel was mostly seen at pipet voltages lower than +30 mV. The lower trace shows the activity burst from the middle and upper traces marked with an asterisk. The dashed line in the lower trace represents unitary conductance of 109 pS for this channel and 'C' refers to the closed level of the channel. (B) The channel was more active at positive pipet voltages. At voltages higher then +30 mV the regular bursting behavior disappeared and the channel displayed multiple conductance states. Asterisk marks one of few events of the unitary conductance 109 pS. Note the 3.5-times slower time base then in the upper trace in 'A'. (C) In most patches the channel required a threshold voltage for activation. The threshold voltage was always higher after the patch was being kept at negative pipet voltages – the bottom recording. The upper trace and lower trace were recorded from separate patches.

unitary currents measured in this way were plotted against voltage in Fig. 4A (squares) and the channel conductance was calculated from the slope. At positive pipet voltages

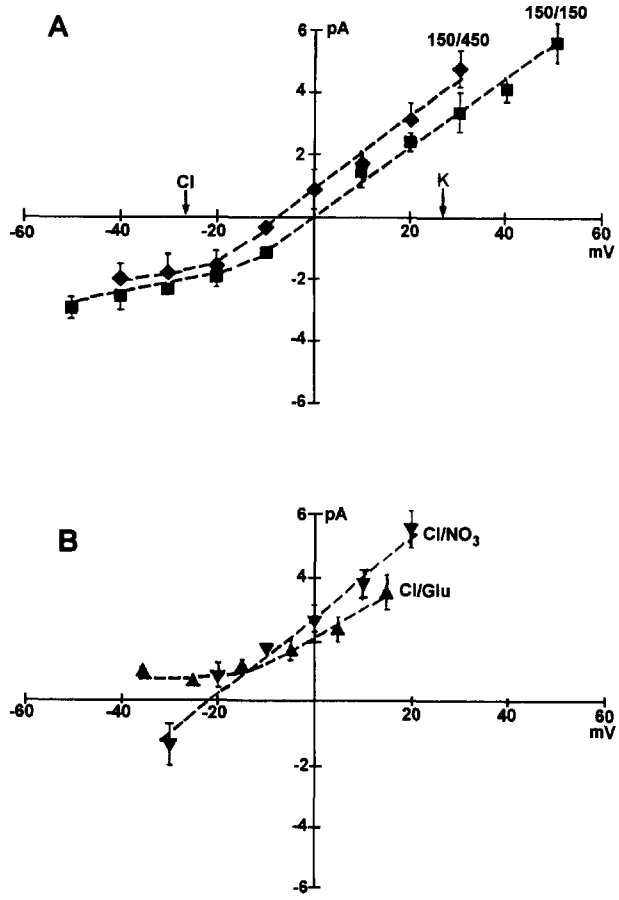

Fig. 4. Current–voltage plots for excised patches. The size of the current steps (means ± S.D.) is plotted against the pipet voltage. (A) $I-V$ plots for symmetric (squares) and asymmetric (diamonds) KCl conditions. 8 mV shift of the asymmetric $I-V$ curve indicates slight preference for chloride over potassium. Arrows marked 'Cl' and 'K' indicate reversal potentials for chloride and potassium respectively. Each point represents 5 to 7 measurements. (B) Single channel currents in 150 mM NO_3^- (inverted triangles) or 150 mM glutamate (triangles) in the bath and 150 mM Cl^- in the pipet. The reversal potential for Cl^-/NO_3^- bi-ionic condition was −22 mV. Each point represents at least 3 measurements. As in 'A', in some cases, the standard deviation lies within the thickness of the symbol.

this unit conductance was found to be 109 ± 14 pS in a symmetric 150 mM KCl, in seven independent studies. In all 37 patches studied the measurements have been done until the bursting behavior disappeared irreversibly. The 109 pS conductance is almost entirely missing in the channel activities at +45 mV shown in Fig. 3B, upper trace. The predominant conductance at +45 mV of this channel in this patch was in the range 25–30 pS, another conductance encountered was 60 pS. There were only few events when the channel (from this patch) dwelled on the 109 pS level, one of them is marked with an asterisk.

As mentioned previously the channel is voltage dependent and it is predominantly active upon positive voltages. Moreover, as it is shown in Fig. 4A there is a rectification of a single unit current–voltage plot upon negative pipette voltages. At negative voltages the conductance of the channel decreased to 49 pS.

3.5. Ion preference

The ion preference of this channel was investigated by changing the KCl concentration in the bath from 150 mM (symmetric solutions of both sides of the membrane) to 450 mM (asymmetric solutions, 150 mM KCl in the pipet and 450 mM KCl in the bath). This bath change caused a 8 mV left shift of the $I-V$ curve indicating a slight preference of Cl^- over K^+ (Fig. 4A). Equimolar substitution of the bath Cl^- with NO_3^- shifted the reversal potential from 0 to −22 mV which indicates a preference for nitrate over chloride under these conditions (Fig. 4B). From the shift of the reversal potentials under the bi-ionic conditions, the selectivity sequence of the channel calculated using the Goldman-Hodgkin-Katz equation was $P_{NO3}/P_{Cl}/P_K$ = 2.7:1.0:0.4. When the bath was perfused with a solution containing glutamate it was not possible to detect a reversal potential within the voltage range tested (Fig. 4B).

3.6. Channel blockage

In five independent experiments, it was found that anthracene-9-carboxylic acid (9-AC), a powerful Cl channel blocker [19], was effective in blocking these conductances in *E. coli* protoplasts patches (Fig. 5). 300 μM of 9-AC was sufficient to inhibit these activities completely.

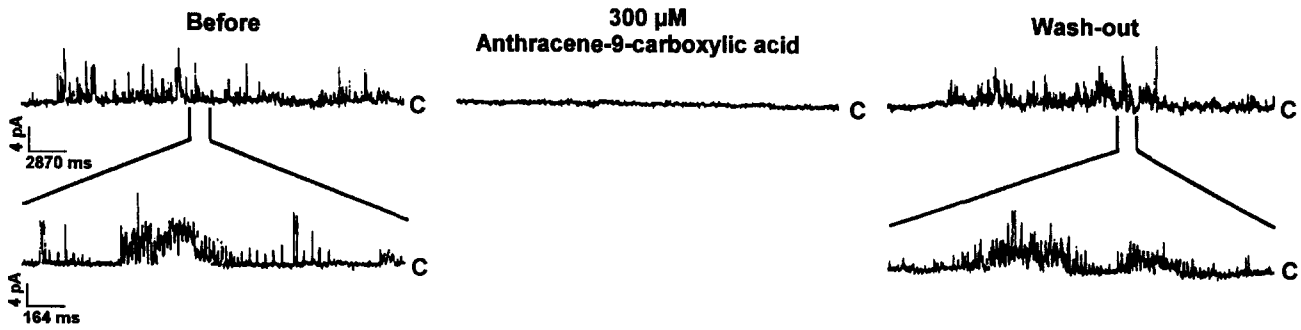

Fig. 5. 300 μM 9-AC completely blocked the channel activities and the perfusion of the same bath solution without 9-AC restored activity. Voltage = +30 mV.

This inhibition was reversible. Activities returned when 9-AC was washed out by perfusion. 9-AC was dissolved in DMSO. In control experiments presence of DMSO in similar concentration in the bath did not affect channel activities.

4. Discussion

The inner membrane of *Escherichia coli* has never until recently [11,19,12] been explored with electrodes although some studies have been done on inner membrane fraction separated with a sucrose gradient from the outer membrane and incorporated in asolectin liposome [20–23]. Giant protoplasts, a preparation more closely resembling live cells than reconstituted liposomes, were used in this patch-clamp study and the studies by Berrier et al. [19] and Cui et al. [12]. Berrier et al. [19] obtained the protoplasts suitable for patch-clamping from the strains of *E. coli* that grow into giant round cells. They removed the outer membrane from the giant cells in the way similar to that described in this paper, by EDTA-lysozyme treatment combined with osmotic shock. Application of patch-clamp technique to giant bacterial protoplasts enables physiological studies of function of various transport proteins in their natural environment. It has also another advantage over the reconstituted system: since perfect separation of inner and outer membranes by gradient centrifugation cannot be achieved [24] it leaves no doubt that the channels under investigation are localized in the inner membrane indeed.

The discovery of ion channels in the cytoplasmic membrane of *E. coli* does not confirm a claim that bacterial cells, unlike eucaryotic cells, do not have ion channels, except in the outer membrane [25]. This view originated in a belief that since the inner membrane sustains the proton motive force (PMF) it should not be leaky and contain pores that pass ions quickly. A small size of bacterium and a high rate of ion transport through an ion channel may place bacteria at risk of an uncoupling. Since ion channels exist in the inner membrane the PMF may not be preserved at all times and the inner membrane can be temporarily electrically discharged. On the other hand ion channels tend to inactivate themselves and can be activated under certain physiological conditions only. The channels detected under laboratory conditions may remain silent or inactivated in vivo. The anion channel described in this paper is a good example of a channel that is very likely to remain in its inactivated state most of the time.

The anion channel from *E. coli* inner membrane is highly regulated. It does not activate unless certain positive pipet voltage is applied to the patch and it is less active upon negative pipet voltages. Similar observations have been reported for maxi chloride channels [18] – a class of anion channels – from other preparations [26–28].

An interesting feature of this channel is its bursting, oscillatory behavior exhibited during short period after activation. After several minutes this behavior disappears and irreversible degradation of the channel to multiple subconductance levels has been observed. The number of conductance states adopted by the channel does not appear to be fixed, either between separate channels under the same conditions, or in one channel at different holding potentials. Such gating has been displayed by another multistate anion channel from *Amaranthus* protoplasts [30] and large number of conductances (up to 16 [27]) has been reported for most maxi chloride channels.

The *E. coli* channel inactivation in time raises the possibility that the channel degradation and/or its assembly might be under control of some intracellular factor. This possibility was not tested in cell attached patches, since the presence of glutathione on the cytoplasmic side of the membrane patch was a prerequisite for the stability of the GOhm seal. Glutathione is a tripeptide (cysteine + glycine + glutamic acid) which reduces cysteine SH-groups in peptides and effects the conformation of polypeptides. A possibility that the observed behavior of the *E. coli* channel can be attributed to glutathione can not be ruled out.

The selectivity sequence, $NO_3^- > Cl^- > K^+$ indicates that the channel discriminates by charge but the permeability ratios indicate slight preference for anions over cations. A high permeability of nitrate ions relative to chloride ions is similar to other chloride channels (e.g., [29,30]).

An analog of carboxylic acid, 9-AC could reversibly block the channel when applied to the bath side of the membrane patch. A 300 μM dose of 9-AC was sufficient to abolish the current completely. In other preparations usually higher concentrations (1–4 mM) of the blocker were effective (e.g. [31,32]).

The channel described here possesses some of the characteristics of the maxi chloride channels. Physiological role of maxi chloride channels is, in general, unclear. They may contribute to the resting Cl conductance and regulate cell volume (for review see: [18]). In *Escherichia coli* the anion selective channel may be involved in salt efflux. As proposed for mitochondrial IMAC (inner membrane anion channel) by Beavis [33] the electrophoretic translocation of anions may be driven by membrane potential while electroneutral efflux of K^+ via K^+/H^+ antiporter is driven by pH. The channel opens when the membrane potential is near zero or the cytoplasmic side of the membrane patch is slightly negative. Since membrane resting potential in bacteria is around -130 mV [34] it is possible that the channel remains silent until an emergency associated with a sudden drop of membrane potential.

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