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Electrophysiological Studies in *Xenopus* Oocytes for the Opening of *Escherichia coli* SecA-Dependent Protein-Conducting Channels

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Abstract. Protein translocation in *Escherichia coli* requires protein-conducting channels in cytoplasmic membranes to allow precursor peptides to pass through with adenosine triphosphate (ATP) hydrolysis. Here, we report a novel, sensitive method that detects the opening of the SecA-dependent protein-conducting channels at the nanogram level. *E. coli* inverted membrane vesicles were injected into *Xenopus* oocytes, and ionic currents were recorded using the two-electrode voltage clamp. Currents were observed only in the presence of *E. coli* SecA in conjunction with *E. coli* membranes. Observed currents showed outward rectification in the presence of KCl as permeable ions and were significantly enhanced by coinjection with the precursor protein proOmpA or active LamB signal peptide. Channel activity was blockable with sodium azide or adenylyl 5′-(β,γ-methylene)-diphosphonate, a nonhydrolyzable ATP analogue, both of which are known to inhibit SecA protein activity. Endogenous oocyte precursor proteins also stimulated ion current activity and can be inhibited by puromycin. In the presence of puromycin, exogenous proOmpA or LamB signal peptides continued to enhance ionic currents. Thus, the requirement of signal peptides and ATP hydrolysis for the SecA-dependent currents resembles biochemical protein translocation assay with *E. coli* membrane vesicles, indicating that the *Xenopus* oocyte system provides a sensitive assay to study the role of Sec and precursor proteins in the formation of protein-conducting channels using electrophysiological methods.

Key words: Protein-conducting channel — SecA — *E. coli* inverted membrane vesicle — Signal peptide — Voltage clamp — *Xenopus* oocyte

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Introduction

Bacterial protein translocation across cytoplasmic membranes has been studied extensively in *Escherichia coli* (Cabelli et al., 1988; Manting & Driessen, 2000; Mori & Ito, 2001; Veenendaal, van der Does & Driessen, 2004; Vrontou & Economou, 2004; Watanabe & Blobel, 1993). Preproteins are primarily transported from the cytoplasm to the periplasm by protein translocase. It is widely accepted in the prevailing model that the core of preprotein translocase is embedded in inner membrane and comprised of SecYEG complexes in *E. coli* (Breyton et al., 2002; Manting et al., 2000; Meyer et al., 1999; Mitra et al., 2005; Wickner & Leonard, 1996). Their conserved homologues, Sec61 complexes, play this role in eukaryotes (Gorlich et al., 1992; Hanein et al., 1996; Jungnickel, Rapoport & Hartmann, 1994). SecA, a critical component of the protein translocation machinery, cycles on and off to interact with SecYEG by insertion of a 30-kDa carboxyl-terminal domain (Economou & Wickner, 1994) into the protein-conducting channel, forming a heterotetrameric complex during preprotein translocation and consequently translocating preproteins in an adenosine triphosphate (ATP)-dependent manner (Manting et al., 2000; Uchida, Mori & Mizushima, 1995; van der Wolk, de Wit & Driessen, 1997). This 901-amino acid protein (Schmidt et al., 1988) was initially characterized as being both a soluble and a peripheral membrane protein (Cabelli et al., 1991; Oliver & Beckwith, 1982). More recent studies, however, have shown that it behaves as an integral membrane protein (Chen, Brown & Tai, 1998; Chen, Xu & Tai, 1996) upon interaction with lipids and as an integral part of the protein-conducting channel.

Most biochemical analyses of protein translocation across membranes have relied heavily on the

acquisition of protease resistance by translocating proteins into membrane vesicles. Electrophysiological studies have indicated that protein translocation across membranes occurs through protein-conducting channels in both prokaryotes and eukaryotes (Simon & Blobel, 1991, 1992; Simon, Blobel & Zimmerberg, 1989; Wirth et al., 2003). Using these methods, Wirth et al. (2003) studied the channel properties of mammalian Sec61p complex mixed with proteoliposome to form lipid bilayer. Simon and Blobel (1992) demonstrated that signal peptides open the protein-conducting channels, leading to ion fluxes across the membranes fused with *E. coli* membrane vesicles and protoplasts with planar lipid bilayers and opening the protein-conducting channels. However, Sec proteins have not been identified in their studies. It was reported that protein translocation renders *E. coli* membranes permeable to halide anions (Schiebel & Wickner, 1992) and to the countermovement of protons (Kawasaki, Mizushima & Tokuda, 1993). Therefore, if these Sec protein-conducting channels are permeable to ions, there should be a way to identify them and measure the ion fluxes across cytoplasmic membranes during the opening of the channels. Moreover, whether the Sec proteins can indeed increase the ion fluxes has not been demonstrated.

Oocytes from *Xenopus laevis* are a reliable and widely used system for analyzing ion channels and transporters (Koren, Burstein & Soreq, 1983; Soreq & Seidman, 1992; Zhu et al., 1999). These cells have several advantages for electrophysiological measurements of substance translocating across membranes and are routinely used in such studies. Their size, availability and stable expression of exogenous proteins allow a wide range of manipulations of foreign components, electrode penetrations for low noise and long-lasting electrophysiological studies. Thus, the *Xenopus* oocyte system, if feasible, may be a useful addition for studying certain aspects of protein translocation.

In this study, we develop a novel and sensitive method for the study of activity of *E. coli* protein-conducting channels in *Xenopus* oocytes using electrophysiological techniques. We found that SecA is essential for the formation of protein-conducting channels in this system. Injection of *E. coli* inverted membrane vesicles into *Xenopus* oocytes produced outward ionic currents but not by SecA-depleted *E. coli* membranes. These currents were strongly enhanced upon addition of SecA and precursor proteins or signal peptides. The channel activity was completely blocked by sodium azide or a nonhydrolyzable ATP analogue, both of which are known to be effective inhibitors of SecA protein. These results show that the currents detected in oocytes report on SecA-dependent protein translocation in bacteria. Such methods will provide a useful sensitive assay for further dissecting the components and properties of protein-conducting channels.

Materials and Methods

Bacterial Strains

All strains used are *E. coli*. BA13 is a *secA13(am)supF(ts)* mutant of K12 strain MC4100 (F^- *lacU169 araD136 relA*$^-$ *rpsL150 febB5301 deoC7 ptsF25 thi*$^-$) (Emr & Silhavy, 1983). BL21 (λDE3)/pT7-SecA overproduces SecA when induced (Cabelli et al., 1988). Cells were grown in a buffered Luria-Bertani medium (Tai et al., 1991) with 0.5% glucose with aeration.

Reagents and Chemicals

All chemicals are reagent-grade and were obtained from Sigma-Aldrich (St. Louis, MO) or other commercial sources.

Purification of SecA, ProOmpA and LamB Signal Peptides

SecA was purified from BL21 (λDE3)/pT7-SecA as described (Cabelli et al., 1988). Purified proOmpA, wild-type LamB signal peptide (MMITLRKLPLAVAVAAGVMSAQAMA) and LamB deletion mutant signal peptides (MMITLRKLP—VAAGVMSAQAMA) were prepared as described (Chen et al., 1987, 1996). Protein amounts were determined using a Bio-Rad (Richmond, CA) assay kit with bovine serum albumin as a standard.

Preparation of Membranes

SecA-depleted membrane vesicles were prepared from BA13 cells as described (Tai et al., 1991). Briefly, BA13 cells were grown to mid-log phase at 30°C, then diluted to $0.1A_{600}$ unit and shifted to 42°C until growth ceased due to depletion of SecA. The cells were harvested and taken through the standard membrane preparation procedures (Tai et al., 1991). SecA-containing wild-type MC4100 membranes were prepared as described (Tai et al., 1991).

Xenopus Oocyte Preparation and Injection

Oocytes were obtained from the frog *X. laevis* (Mao et al., 2004; Xu et al., 2001). Frogs were anesthetized by bathing in 0.3% 3-aminobenzoic acid ethyl ester. A small abdominal incision (~5 mm) was made near the underbelly of the frog, and a few lobes of ovaries were removed. Then, the surgical incision was closed, and the frog was allowed to recover from the anesthesia. *Xenopus* oocytes were treated with 2 mg/ml of type IA collagenase in OR2 solution (82 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$ and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.4) for 60 min at room temperature, followed by three washes (10 min each) of the oocytes with the OR2 solution. The oocytes were maintained in 16°C for 2–4 days in order to obtain stable and constant results.

SecA and *E. coli* membranes with or without proOmpA or LamB signal peptides were mixed with gentle vortexing in TK buffer (10 mM Tris-HCl [pH 7.6] and 50 mM KCl) and incubated at 30°C for 4 min. HEPES (20 mM) in TK buffer was used when samples were injected with puromycin. Then 50-nl samples in various concentrations were injected into the dark animal pole of the oocytes using a borosilicate glass pipette (1.2 mm outside diameter, 0.69 mm inside diameter; Sutter Instrument, Novato, CA) connected to a Nanoject II injector (Drummond Scientific, Broomall, PA). The glass pipettes were pulled from a Flaming/Brown Micropipette Puller (model P-97, Sutter Instrument). The tip was trimmed to 15–18 μm in inner diameter. The oocytes were

then incubated at 23°C in ND-96 buffer (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 5 mM HEPES, 40 mg/l sodium pyruvate and 100 mg/l geneticin [pH 7.4]) with tetracycline (100 mg/l) to prevent bacterial growth in the buffer solution. The standardized conditions were injections of 60 ng membranes, 20 ng SecA, 0.28 pmol proOmpA and 0.3 pmol LamB signal peptides and recorded after 2 h unless specific amounts and time were applied. Effective concentration of chemicals in the oocytes was estimated based on the average volume of oocytes at 500 nl with 50 nl injection.

Voltage-Clamp Measurements

We adopted an electrophysiological method, i.e., the voltage clamp, to measure the opening of protein-conducting channels. This method is based on the principle that ionic currents can be recorded when ions pass through the cell membranes. The plasma membranes are impermeable to ions unless there are openings or channels in the membranes. The protein-conducting channels thus may serve as a pathway allowing ions across the membranes. More importantly, the activity of the protein-conducting channels may be measured, e.g., in the presence of signal peptides.

Currents through the plasma membranes of the oocytes were measured after the oocytes were injected with tested materials. The cells were placed in a recording chamber (model BSC-HT, Medical System corp., Greenvale, NY) on a supporting nylon mesh so that the perfusion solution bathed both the top and the bottom surfaces of the oocytes. Two-electrode voltage clamping was performed using an amplifier (Geneclamp 500; Axon Instruments, Foster City, CA) at room temperature (23–25°C). Cells were impaled using electrodes filled with 3 M KCl (schematically presented in Fig. 1A). One of the electrodes (1.0–2.0 MΩ) used for voltage recording was connected to the HS-2 × 1L headstage (input resistance 10^{11} Ω), and the other electrode (0.3–0.6 MΩ) was used for current recording to the HS-2 × 10 MG headstage (maximum current 130 μA). The electrode was connected through a silver wire that was chloridized freshly for each experiment. For the reversal potential measurements, the chloridized grounding electrode was placed in an agar bridge (5% agar in 3 M KCl). Oocytes were used for further experiments only if their leak currents, measured as the difference before and after a leak subtraction, were $< 10\%$ of the peak currents. The leak subtraction was not applied for data acquisition and analysis. Current records were low pass-filtered (Bessel, 4-pole filter, 3 db at 5 kHz), digitized at 5 kHz (12-bit resolution) and analyzed using pClamp6 (Axon Instruments). The highest and lowest records were eliminated, and data are presented as means ± standard error (SE, n for number of oocytes).

Ion permeability was measured by reversal potential and the current amplitude when one ion species in the bath solution was substituted for K^+ or Cl^-. To minimize the junction potentials, extracellular chloride was only partially replaced, while all extracellular K^+ was substituted with interested cations.

Results

Ionic Currents Can Be Detected upon Injection of *E. coli* Wild-Type Membrane Vesicles

Studies were performed in *Xenopus* oocytes using a two-electrode voltage-clamp technique after various *E. coli* membrane vesicles had been injected (Fig. 1A). Whole-cell ionic currents were recorded 30–180 min after samples were injected into *Xenopus* oocytes. At 150 mV, the current amplitude averaged 6.0 ± 0.3 μA (n = 10, Fig. 1D). The currents were not seen in oocytes injected with the same volume of water or a buffer solution (n = 10, Fig. 1B, 2). Also, membrane currents were not observed until 1.5 h after injection. After that time, the amplitude of the current increased sigmoidally and then reached saturation in 2.5 h (Fig. 1C).

The amplitude of the currents was dependent on signal peptides or precursor proteins. Injection of MC4100 membranes together with LamB signal peptides and SecA resulted in ionic currents similar to those recorded with proOmpA in a time course experiment, and these currents (5.2 ± 0.2 μA, n = 8) were at a similar level to those from addition of proOmpA (5.0 ± 0.4 μA, n = 6, Fig. 1C).

The currents produced were also dependent on the concentration of MC4100 membranes injected. Detectable currents were seen with injection of 20 ng MC4100 membranes; larger currents were produced upon addition of increasing amounts of MC4100 membranes (Fig. 1D). The effect was also saturable, suggesting limiting sites or components of the interaction.

To assess the specific contribution of SecA to the membrane currents, SecA was injected with various amounts of MC4100 membranes. Compared to results from addition of MC4100 membranes alone, the SecA-dependent currents were much greater, reaching a maximum of 7.8 ± 0.1 μA (n = 9, Fig. 1D). Effects of the natural substrates of translocation, namely, precursor proteins, were examined with proOmpA, the precursor of outer membrane protein A. With proOmpA injected together with MC4100 membranes and SecA, the induced currents were 8.0 ± 0.1 μA (n = 6, Fig. 1D) and reached a maximum amplitude faster than with MC4100 membrane alone or with MC4100 membrane plus SecA, indicating that the stimulation of channel activity is likely to result from the binding of proOmpA to SecA in the membrane, thereby opening the channel.

Various amounts of purified SecA were injected alone or with MC4100 membranes (20 ng) with or without proOmpA. The resulting currents were observed 2 h after injection and reached a maximum of 3.0 ± 0.2 μA (n = 10, Fig. 1E). Moreover, the increase in the amplitude of currents was more evident in the presence of proOmpA (4.9 ± 0.3 μA, n = 7, Fig. 1E).

SecA Protein Plays a Critical Role in the Production of Ionic Currents

The wild-type MC4100 membranes contained significant amounts of SecA. The roles of SecA were therefore explored using membranes depleted of SecA at 42°C. Various amounts of SecA-depleted *E. coli* BA13 membranes were injected into *Xenopus* oocytes. The ionic currents shown in MC4100 membrane

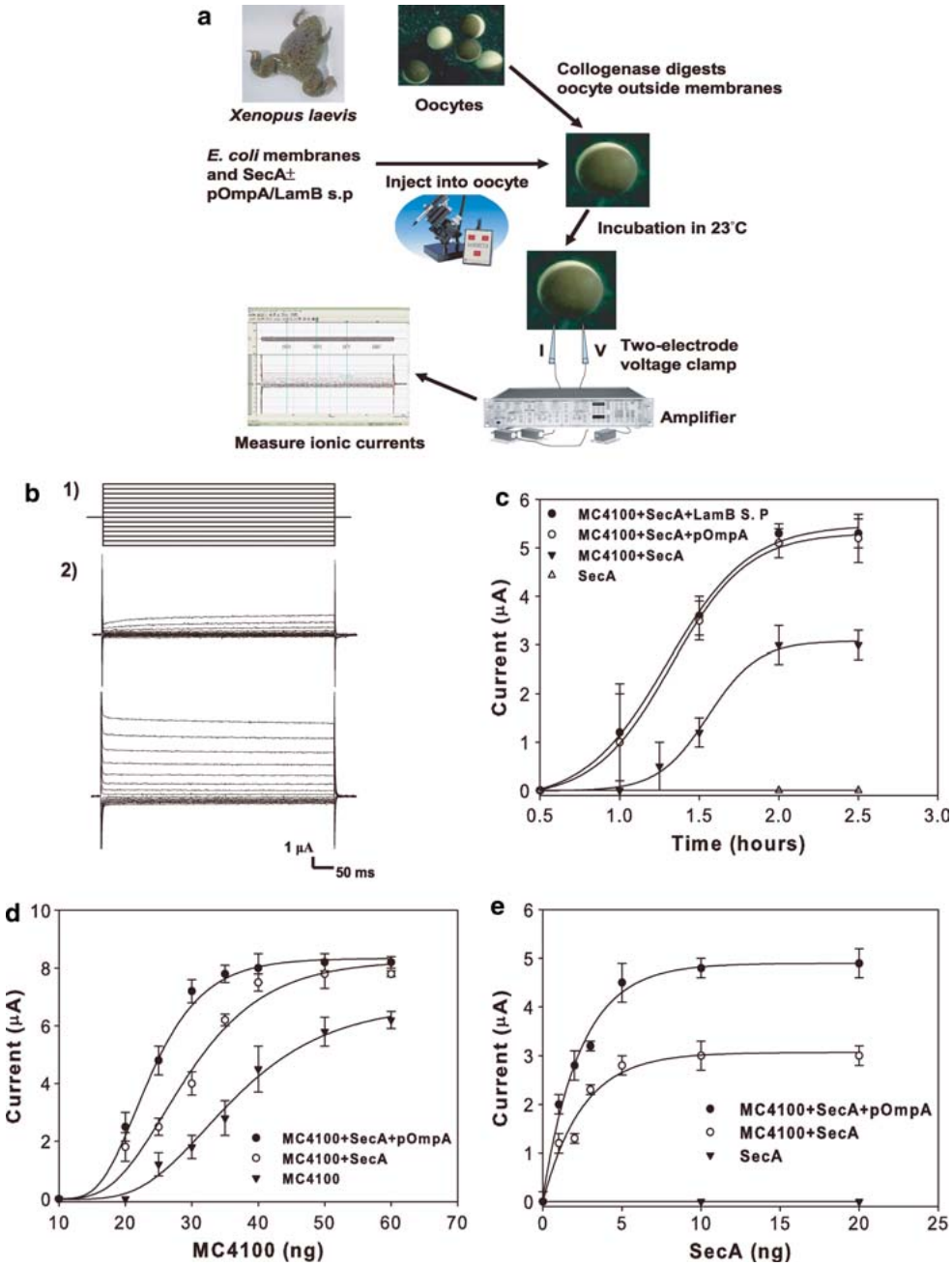

Fig. 1. Channel activities of wild-type MC4100 membrane. (*A*) Schematics of the *Xenopus* oocyte system. Currents were recorded with the voltage-clamp technique after injection. (*B*) Current recordings. (*1*) Membrane potential (V_m) was held at 0 m*V* in the presence of KCl (90 mM) in the bath solution, and a series of command pulse potentials from −150 to 150 m*V* with a 20-m*V* increment was applied to the oocytes. (*2*) With TK buffer injection, small currents were recorded (*upper*). The current amplitude was significantly larger with a completely different trajectory with injection of MC4100 membranes (*lower*). (*C*) Time courses of ionic currents. MC4100 membranes (20 ng) and SecA were mixed with or without of proOmpA or LamB signal peptide and then injected into oocytes. The currents were recorded at the indicated time. (*D, E*) Different amounts MC4100 membranes (*D*) or SecA (*E*) were injected into oocytes with or without proOmpA.

recordings were not detectable when BA13 membranes alone were injected (Fig. 2A) unless SecA was added as well. Twenty nanograms of SecA was sufficient to provide maximal current of 7.0 ± 0.1 μA (n = 10, Fig. 2A). Furthermore, the precursor protein proOmpA increased and stimulated the ionic currents to higher amplitude (7.8 ± 0.3 μA, n = 7, Fig. 2A). When saturating amounts of SecA were added with BA13 membranes, the stimulated ionic current activities were similar to those observed with MC4100 membranes (Fig. 2A), with respect to both the extent of the stimulation and the dependence on the amount of membranes.

As little as 5 ng SecA along with BA13 membranes stimulated the ionic current to the saturation level of 2.8 ± 0.2 μA (n = 9, Fig. 2B). In the presence of BA13 membranes and proOmpA and LamB signal peptides, the ionic current amplitudes were approximately doubled to 4.3 ± 0.6 and 4.5 ± 0.3 μA, respectively (n = 8 and n = 12, Fig. 2B). The ionic current activities require both *E. coli* membranes and SecA; neither BA13 membranes nor SecA alone have any activity (Fig. 2). These results showed that SecA is essential for the currents detected in oocytes with injected *E. coli* membranes.

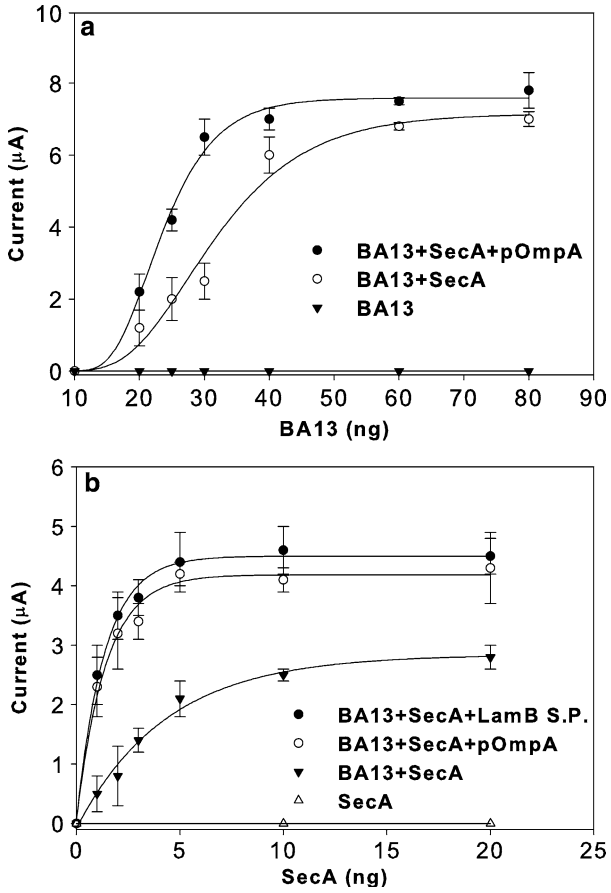

Fig. 2. Dependence of SecA for channel activity in SecA-depleted BA13 membranes. (*A*) No ionic currents could be recorded in the presence of BA13 membranes alone unless SecA or SecA with proOmpA was jointly injected. (*B*) Functional signal peptides or proOmpA enhanced ionic currents only in the presence of SecA.

Roles of Signal Peptides: Effects of Oocyte-Endogenous Precursor Proteins Are blocked by puromycin

Signal peptides were reported to induce the opening of transmembrane protein-conducting channels (Simon & Blobel, 1992). However, ionic currents were observed here in the presence of either MC4100 membranes or BA13 membranes with SecA without adding LamB signal peptides or proOmpA (Figs. 1C and 2A). We reasoned that oocyte-endogenous signal peptides present on precursor proteins may contribute to SecA-dependent channel activity. Thus, puromycin, an efficient protein synthesis inhibitor for eukaryotic cells, was used to remove any newly synthesized proteins containing signal peptides. By coinjecting puromycin (3 mM) with MC4100 and SecA, the ionic currents were almost undetectable (Fig. 3A), while the induced currents were still seen if proOmpA or LamB wild-type signal peptides were present simultaneously (Fig. 3A, C), indicating the direct contribution from proOmpA or LamB wild-type signal peptides. Injection of various amounts of proOmpA or LamB wild-type signal peptides in the presence of puromycin showed a concentration-dependent effect (Fig. 3B, D).

Validation of the System: Effects of Inhibitors of Protein Translocation on Channel Activity

Evidence for the validity of the system is the dependence on ATP hydrolysis by SecA. Optimal protein translocation requires both ATP hydrolysis and a proton motive force, although the latter is not absolutely essential (Chen & Tai, 1985, 1986a; Geller, Movva & Wickner, 1986; Mizushima & Tokuda, 1990). To further demonstrate that SecA is involved in the channel activity observed in oocytes, we tested the effect of ATP hydrolysis using AMP-PCP, a nonhydrolyzable analogue of ATP and known competitive inhibitor of SecA/ATP hydrolysis (Chen & Tai, 1986b). The ion current was blocked completely by adenylyl 5′ (β, γ-methylena)-diphosphonate (AMP-PCP) (4 mM) in the presence or absence of proOmpA with MC4100 membranes (Fig. 4A).

Sodium azide is known to effectively inhibit the transport of proteins across the bacterial cytoplasmic membrane by acting on SecA; therefore, we tested its effect on ion currents in the system. The inhibitory effects of azide are concentration-dependent (Fig. 4B). In the presence of sodium azide (4 mM), complete inhibition of the channel activity was observed when proOmpA was injected alone with MC4100 membranes (Fig. 4B). These data also suggested that SecA formed an azide-sensitive channel in bacterial patches of the oocyte membrane and that the protein-conducting channel activity observed here is indeed SecA-dependent.

Protein-Conducting Channels are Nonselective with More Permeability to Smaller Ions

Ion permeability of the SecA-induced channels was studied with different cations and anions: K^+ (90 mM), Na^+ (90 mM), *N*-methyl-D-glucamine ($NMDG^+$, 90 mM), glutamate$^-$ (80 mM) and gluconate$^-$ (80 mM). The ionic currents were recorded in the presence of MC4100 membranes, SecA and functional LamB signal peptides. Under the voltage clamp, the reversal potential was measured first at baseline. After one of the conductive ions in the bath solution was replaced with another cation or anion, the measurement of reversal potential was repeated. According to the direction of ionic movements, inward currents were studied for cation substitution, while outward currents were examined for anion replacement. When the extracellular solution contained 90 mM KCl, the currents had a reversal potential of -20.2 ± 2.0 m*V* ($n = 8$, Fig. 5A) and an inward K^+ current amplitude of 1.5 ± 0.1 μA ($n = 8$, Fig. 5B). Complete

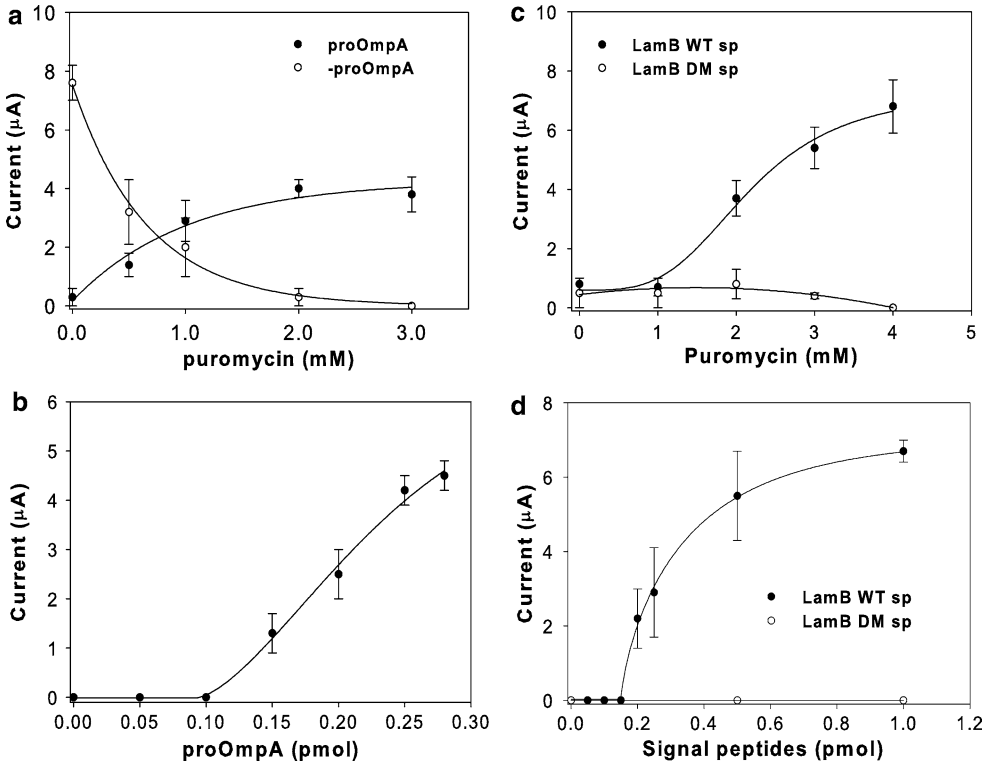

Fig. 3. The effect of signal peptides. (*A*) Inhibition of endogenous precursor protein activity by puromycin. MC4100 membranes and SecA were injected with or without proOmpA. (*B*) Different amounts of proOmpA mixed with MC4100 membranes and SecA were injected in the presence of puromycin (3 mM). (*C*) LamB wild-type (WT, 0.5 pmol) and deletion mutant (DM) signal peptides and (*D*) different amounts of LamB WT and DM signal peptides in the presence of puromycin (4 mM) were injected with MC4100 and SecA.

replacement of the extracellular K^+ with Na^+ had a modest effect on the reversal potential of −21.0 ± 2.1 m*V* (n = 8) and the inward currents (1.1 ± 0.3 μA, n = 8). Substitution of the extracellular cation with $NMDG^+$ shifted the reversal potential to −27.2 ± 2.1 m*V* (n = 8) and reduced the inward current amplitude to 0.4 ± 0.02 μA (n = 8). Since the extracellular Cl^- concentration remained the same, these results indicate that the SecA-dependent channels are nonselectively permeable to cations with lower permeability to large cations such as $NMDG^+$. Similar ion substitution experiments were performed for Cl^-. In this experiment, 10 mM Cl^- remained in the extracellular solution. Upon replacement of 80 mM extracellular Cl^- with 80 mM glutamate$^-$, the reversal potential was shifted rightward by 5.8 ± 2.1 m*V* (n = 8) and the outward current amplitude was lowered by 7.0 ± 0.4 μA (n = 10, Fig. 5C, D). Substitution with 80 mM gluconate$^-$ shifted the reversal potential by 10.3 ± 1.1 m*V* (n = 8) and reduced the current amplitude by 4.3 ± 0.3 μA (n = 10, Fig. 5C, D). Since the extracellular K^+ concentration remained the same, these results indicated that the SecA channels are more permeable to small anions than large ones such as glutamate or gluconate.

The permeability of a given ion was studied by measuring its permeability ratio to K^+ or Cl^-. The relative permeability ratio was calculated according to the Nernst equation: $E_{rev} = \frac{RT}{zF} \ln \frac{P_A[A]_0}{P_B[B]_0}$, where the gas constant R = 8.3145 J • mol^{-1} • K^{-1}, T is absolute temperature, z is the charge of the ion, F (Faraday constant) = 96,500 C • mol^{-1}; experiments were conducted at room temperature of 24°C (297K). The molecular weights in daltons (Da) of these cations and anions were as follows: K^+ (39.1 Da), Na^+ (23.0 Da), $NMDG^+$ (195.2 Da), Cl^- (35.5 Da), glutamate$^-$ (147.1 Da) and gluconate$^-$ (198.8 Da). The ion permeability ratios of Na^+ and $NMDG^+$ versus K^+ were 0.93 and 0.73. Glutamate$^-$ and gluconate$^-$ versus Cl^- in the ion permeability ratios were 0.80 and 0.72, respectively. K^+ had almost the same permeability as Na^+ (the ion sizes with hydration have less relevance to ion permeability in this protein-conducting channel). In comparison, the permeability to all organic ions was significantly lower. These results suggest the following permeability order: $K^+ \geq Na^+ > NMDG^+$ and $Cl^- >$ glutamate$^-$ > gluconate$^-$.

Discussion

We have demonstrated the utility of a novel method to explore *E. coli* channel properties in a physiologically relevant and experimentally tractable system. The electrophysiological method developed in the present study has several advantages over conventional protein biochemical techniques. (1) The method is extraordinarily sensitive. It is known that several electrophysiological techniques are highly sensitive for measurement of ion channel activity, with some of them available for detection of single molecule activity such as patch clamp. Using the

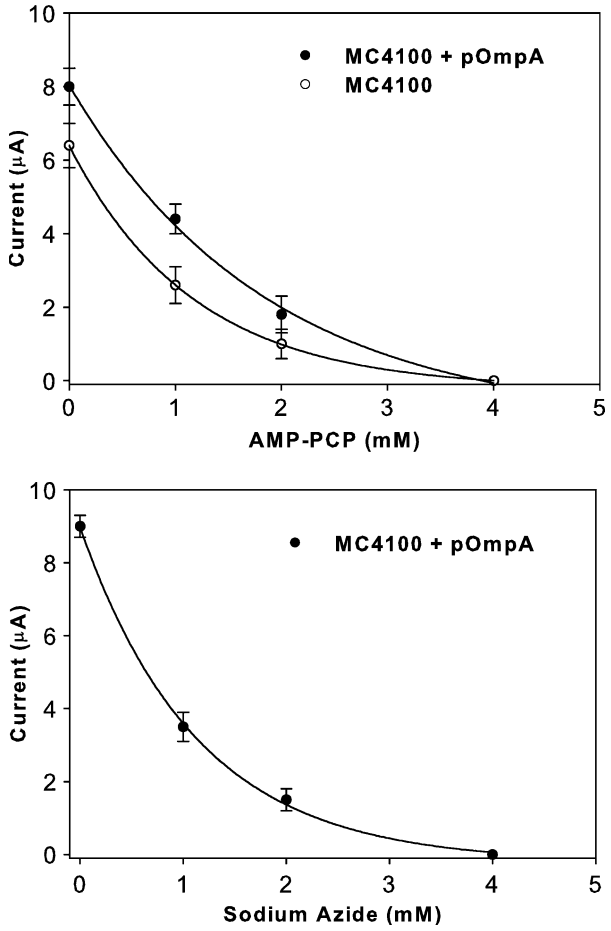

Fig. 4. Effects of AMP-PCP and azide on membrane currents. (*A*) AMP-PCP was added with or without proOmpA in the presence of MC4100 membranes. (*B*) Inhibition of ion current by sodium azide in the presence of MC4100 plus proOmpA.

electrophysiological method, we have observed activity of the Sec channels with an injection of nanograms of proteins. (2) The electrophysiological method allows detection of functional status, i.e., opening and closing, of protein-conducting channels. Our data have shown that the channels are active in the presence of signal peptide and inhibited by SecA protein inhibitors. (3) With this new method, studies of the population activity of protein-conducting channels become possible so that the whole-cell recording from voltage clamp serves our experimental purpose.

We have shown the dynamics of channel activity in *Xenopus* oocytes, the dose dependence on wild-type LamB signal peptides and the changes of the dose-response relationship in the presence of puromycin. However, we cannot fully demonstrate the mechanisms of the injected *E. coli* membranes on the *Xenopus* oocyte plasma membrane. The observed currents in *Xenopus* oocytes following injection of *E. coli* membranes with or without SecA proteins, proOmpA or LamB signal peptides can be produced by two means: the opening of protein-conducting channels gives rise to the ionic currents and, alternatively, the materials injected might stimulate the expression of oocyte-endogenous ion channels in the plasma membranes. We do not believe that the latter is the case because the channel activity is augmented by signal peptides and suppressed by SecA protein inhibitors. Therefore, it is very likely that the ionic currents are conducted through the protein-conducting channels. We were not able to demonstrate the mechanisms of injected *E. coli* membranes on the *Xenopus* oocyte plasma membrane. Indeed, we attempted to use the conventional biochemical and morphological methods using fluorescent labels to examine the fate of bacterial membranes and SecA after injection into oocytes. These methods so far have proved to be not sensitive enough to convincingly demonstrate the mechanism. On the other hand, the electrophysiological voltage-clamp method is based on the principle that ionic current can be recorded when ions pass through the cell membrane. With this new and sensitive method, the protein-conducting channels can be studied as other membrane transports such as ion channels, receptors and transporters, using pharmacological tools. It should be noted that the method detects the opening of the protein-conducting channel and not necessarily the protein translocation. Thus far, we have no evidence that injected proOmpA or proPhoA was secreted. Indeed, the current activity is not directly related to successful translocation of precursors; proPhoA is not active in translocation *in vitro* (Yang, Yu & Tai, 1997b) yet elicited current activity with SecYEG-minus membranes in the oocytes (*unpublished*).

The whole-cell *Xenopus* oocyte system used in our present studies differs from the planar lipid bilayers used in previous work (Simon & Blobel, 1991, 1992; Simon et al., 1989) for single-channel recording of protein-conducting channels of *E. coli* membranes in at least two aspects: (1) these currents were recorded in a cellular environment with intact plasma membranes and (2) the currents are the sum of all channel activity showing trajectories and kinetics more resembling those in the *E. coli* membranes. Using this new approach, we have confirmed the earlier observation (Simon & Blobel, 1992) that synthetic wild-type LamB signal peptides are the physiological ligands that open protein-conducting channels at the initiation of protein translocation across membranes. Using voltage-clamp recording, we have shown that SecA and precursor proteins (or signal peptides) are essential in the detection of ionic currents that arise upon injection of *E. coli* membranes into *Xenopus* oocytes. We show that the ionic currents are completely inhibited by azide (Fig. 4B). These observations are in full agreement with *in vitro* biochemical studies on protein translocation systems,

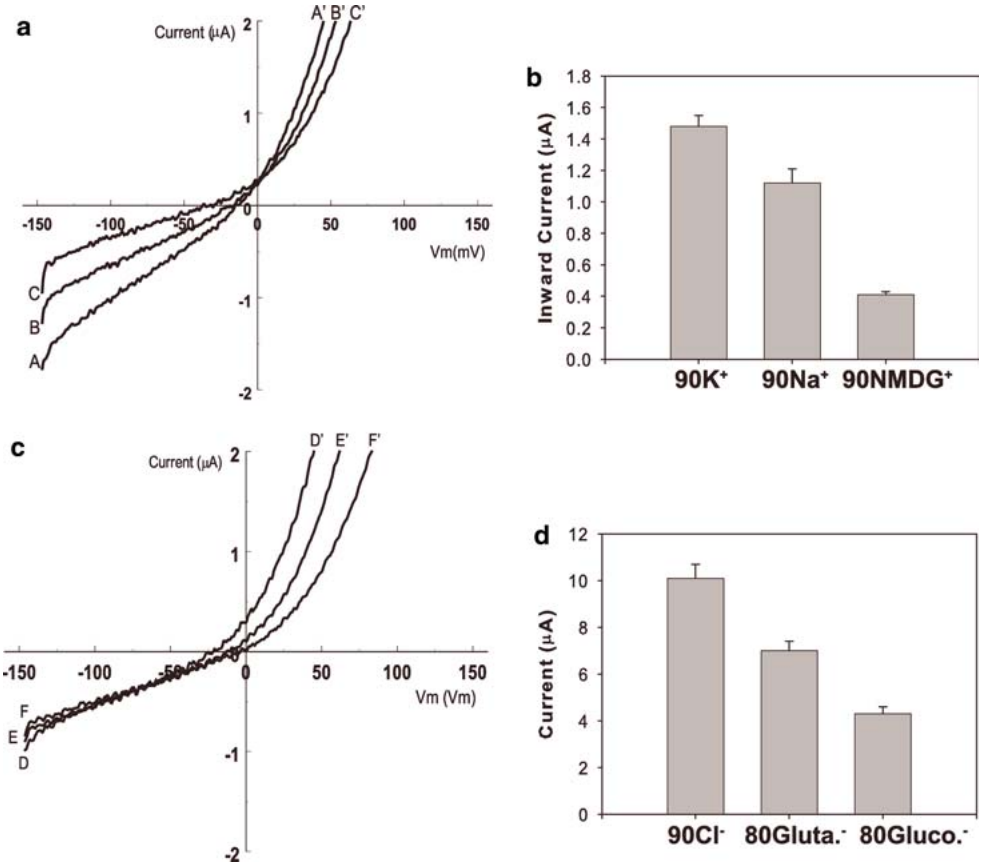

Fig. 5. Ion permeability of protein-conducting channels. (*A*) LamB signal peptides, MC4100 membranes and SecA were injected into an oocyte. Membrane ionic currents were recorded 2 h after the injection; bath solution contained 90 mM K^+. Under such conditions, currents showed a reversal potential of −19.5 m*V* (A-A′). Complete replacement of the extracellular K^+ with the same concentration of Na^+ shifted the reversal potential to −21.0 m*V* (B-B′). Substitution of all K^+ with $NMDG^+$ moved the reversal potential to −27.2 m*V* (C-C′). Note that the Cl^- concentration remained the same in these experiments. (*B*) Ion substitution also caused changes in the amplitude of membrane currents. Inward currents were measured with the same concentration (90 mM) of extracellular KCl, NaCl or NMDG-chloride. (*C*) Similar experiments were carried out by replacing extracellular anion in another oocyte with the same injections. Moderate shifts in reversal potential were seen when 80 mM Cl^- was replaced with the same concentration of gluconate$^-$ (F-F′) or glutamate$^-$ (E-E′) compared with using KD−90 as the bath solution containing 90 mM K^+ (D-D′). The K^+ concentration remained at 90 mM and Cl^- at 10 mM in these experiments. (*D*) The amplitudes of the ionic currents were measured by the outward currents. In recording with various anions, 80 mM Cl^- was replaced with glutamate$^-$ or gluconate$^-$ as in *C*. Gluta.$^-$, glutamate$^-$; Gluco.$^-$, gluconate$^-$.

supporting the notion that the ionic currents reflect protein-conducting channel activity (Chen & Tai, 1985, 1986a; Geller et al., 1986). The effects of ATP analogues and azide further support the roles of SecA and ATP hydrolysis in the process and indicate that the activity does not only reflect the insertion step of SecA in the insertion/deinsertion cycle of SecA in the membranes (Chen et al., 1996). Since SecA/ATP and precursor protein support the channel activity that has the characteristics of protein translocation *in vivo*, i.e., complete inhibition by azide or an ATP analogue, we conclude that the observed ionic current activity reflects the opening of SecA-dependent protein-conducting channels induced by signal peptides or precursor protein. Hence, the new system revealed that the channel openings occur only in the presence of SecA; signal peptides alone, even at high concentration, have no activity in the absence of SecA. These results are consistent with previous genetic, physiological and biochemical studies (Chen et al., 1987; Emr & Silhavy, 1983). Compelling arguments have been made (Schiebel & Wickner, 1992; Simon & Blobel, 1991, 1992; Simon et al., 1989) that the signal peptide binds within the aqueous channel at the mouth of the pore. Our data indicate that this binding could be with SecA, which is known to interact directly with signal peptides (Economou, 1999; Manting & Driessen, 2000).

Moreover, our results have shed light on the ion permeability of these protein-conducting channels. As expected for a protein-conducting channel, the channel activity shows no selectivity for anions or cations but may depend on the size of the ions. Consistent with previous studies (Simon et al., 1989),

our data showed that these channels are less permeable to large anions such as glutamate$^-$ and gluconate$^-$. Interestingly, we have found that these channels are also selective by size for cations. While the channels show a similar permeability to K^+ and Na^+, their permeability to $NMDG^+$ is much smaller. When both reversal potential and current amplitude are considered, the following permeability is suggested in these channels: $Na^+ \geq K^+ > NMDG^+$ and $Cl^- >$ glutamate$^-$ > gluconate$^-$.

Current flow is not detectable with low concentration of SecA under conditions (<1 ng) in which the effects of signal peptides on opening the channel are most pronounced (Fig. 2A, B). That ionic current occurs in the presence of higher amounts of SecA (>5 ng) but in the absence of precursors/signal peptides raises some interesting questions. We assume that oocyte-endogenous precursor proteins carrying signal sequences (Darzacq, Singer & Shav-Tal, 2005; Koren et al., 1983; Mowry & Cote, 1999; Palacios & St Johnston, 2001) also have effects on these protein-conducting channels in *E. coli* membranes. Therefore, puromycin was used to block the synthesis of oocyte-endogenous proteins, thus presenting an environment of free oocyte-endogenous precursors and showing that no ionic currents can be observed in the presence of MC4100 membranes and SecA (Fig. 3). Moreover, these ionic currents can be detected with LamB wild-type signal peptides but not with a nonfunctional deletion mutant of the LamB signal peptides (Fig. 3C, D). However, the recovered ionic currents were smaller than those without puromycin, indicating that, in the absence of puromycin, oocyte-endogenous precursors and added precursors compete to open the protein-conducting channels and that those currents were recorded *in toto* by the voltage-clamp whole-cell recording.

Two different channels, of 60 pS and 220 pS in low- and high-salt conditions, have been shown (Simon & Blobel, 1991, 1992; Simon et al., 1989). Here, we used this semiphysiological oocyte system and measured the ionic currents up to 8–10 μA by injecting SecA, *E. coli* MC4100 membranes and functional LamB signal peptides into oocytes. Based on the whole-cell currents, we calculated that the sum of the channel conductance was approximately 53–67 μS (membrane potential is 150 m*V*), suggesting there were 240,000-1,100,000 protein-conducting channels opened in this scenario. It is worth noting that the oocyte system is remarkably sensitive: only nanogram quantities of membranes, SecA and precursor proteins/signal peptides are needed to generate detectable activities. We also observed ionic currents similar to the recordings of *E. coli* SecA by using different bacterial species of SecA from *Bacillus subtilis*, *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* with *E. coli* SecA-depleted BA13 membranes, all at higher concentrations (B. R. Lin and P. C. Tai, *unpublished*). Thus, the ionic current activity of the oocyte system provides an independent and sensitive assay to study the activity of protein-conducting channels, especially in other bacterial SecA-dependent systems where currently there is no available biochemical translocation assay. These electrophysiological studies of protein-conducting channels should both complement single-channel recordings and biochemical studies that rely on the assays of protease protection of translocated proteins inside membrane vesicles (Tai et al., 1991). Though SecYEG is widely believed to be the protein-conducting channel, it should be noted that protein translocation can occur in the absence of SecYEG (Watanabe, Nicchitta & Blobel, 1990; Yang, Lian & Tai, 1997a; Yang et al., 1997b) and that SecA forms a ring structure upon interaction with lipids and may indeed be the major component of protein-conducting channels (Wang et al., 2003). In this regard, SecYEG-minus membranes in the presence of SecA are still active in eliciting ionic current in this system (*unpublished*), consistent with a more prominent structural role of SecA in protein-conducting channels. The method described here provides a sensitive assay to dissect the nature of protein-conducting channels (Breyton et al., 2002; Mitra et al., 2005; Wang et al., 2003; Yang et al., 1997a, 1997b).

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