Catalyzed Insertion of Proteins into Phospholipid Membranes: Specificity of the Process

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ABSTRACT The process of insertion of intrinsic proteins into phospholipid membranes conjures up the thought of enormous energy barriers but is a routine occurrence in cells. Proteinaceous complexes responsible for protein targeting/translocation/insertion into membranes have been studied intensively. However, the mitochondrial voltage-dependent anion channel (VDAC), can insert into phospholipid membranes by an auto-catalytic process called “auto-directed insertion.” This process results in an oriented insertion of VDAC channels and an increase in insertion rate per unit area of 10 orders of magnitude. Here we report that VDAC catalyzes the insertion of PorA/C1 and KcsA by increasing their calculated insertion rate per unit area by 9 orders of magnitude with no detectable effect on the insertion of α-hemolysin. This was measured as a reduction in the delay before the first insertion of these proteins. Gramicidin and PorA/C1 accelerate the calculated insertion rate per unit area of VDAC by 8 and 9 orders of magnitude, respectively. Only PorA/C1 increases the overall rate of VDAC insertion (50-fold) over the self-catalyzed rate. Our results indicate that catalyzed insertion of proteins into phospholipid membranes does not arise simply from disturbance of the phospholipid membrane because it shows strong specificity.

INTRODUCTION
Channel-forming proteins play a very important role in cells, including metabolite exchange, apoptosis induction, initiation and propagation of electrical signals, etc. How these proteins insert into specific membrane systems has long been of interest. Some channel-forming proteins, such as toxins (Merrill and Cramer, 1990; Zhan et al., 1994), can insert into membranes spontaneously presumably because they encounter small energy barriers, about equal to thermal energy. These ectocytic membrane proteins have evolved their own insertion mechanism. The insertion of endocytic channel-forming proteins is generally thought to be catalyzed by cellular insertion complexes. For these and membrane proteins in general, the insertion mechanisms have been studied in great detail (Dalbey and Kuhn, 2000; Mihara, 2000; Van Geest and Lolkema, 2000).

With few exceptions (e.g., chloride intracellular channel family (Tulk et al., 2000) and anchored proteins), most membrane proteins in the internal membrane system are synthesized in the cytosol and then targeted to their destine membranes by leader sequences or specific recognition sites. Receptors in the internal membrane system can recognize these special regions, leading to the insertion of the proteins into the appropriate membranes.

In the case of mitochondria, most proteins are imported from the cytosol and travel to one of several possible locations: two aqueous compartments and at least two, probably three, membrane compartments. Translocase of outer membrane (TOM) proteins recognize the majority of precursor proteins destined for internalization into mitochondria (Hauke and Schatz, 1997; Neupert, 1997; Pfanner and Meijer, 1997). These proteins are translocated across the mitochondrial membranes through the general insertion pore (GIP), consisting of Tom-40 and ancillary Tom proteins (Hill et al., 1998; Kunkele et al., 1998). However, it has been reported that Tom-20 can directly target newly synthesized voltage-dependent anion-channel (VDAC) proteins into the mitochondrial outer membrane without the main component of GIP, Tom-40 (Schleiff et al., 1999), indicating that the insertion of VDAC channels can bypass the GIP pathway. Other experiments show that VDAC could insert into yeast mitochondria lacking functional receptors (Gasser and Schatz, 1983). Thus an alternate pathway for VDAC insertion into the mitochondrial outer membrane seems to exist.

Experiments on the mechanism of VDAC insertion into mitochondria have identified regions of the protein containing putative sorting signals because deletions or point mutations in these sites interfere with insertion (Smith et al., 1995; Court et al., 1996; Angeles et al., 1999). Interestingly, in vitro insertion of the VDAC1 isoform into VDAC-containing yeast mitochondria occurred to a much higher degree than insertion into mitochondria lacking VDAC1 (Angeles et al., 1999), indicating a higher insertion rate. Because VDAC is not part of the protein insertion/targeting machinery, these observations may indicate that VDAC in the outer membrane facilitates the insertion of VDAC in vivo.

Under defined conditions using purified components, VDAC channels have been found to have their own mechanism of insertion into phospholipid membranes by a process called auto-directed insertion: VDAC molecules already in the membrane direct the orientation of uninserted VDAC proteins and accelerate the insertion rate by 10 orders of magnitude (Zizi et al., 1995; Xu and Colombini,
1996, 1997) over the rate of insertion into an unmodified membrane.

This auto-directed insertion was first proposed by Zizi et al. (1995) to explain the asymmetrical voltage-dependent behavior of a population of VDAC channels with specific point mutations. The substitution of glutamate for lysine at positions 145 and 152 of yeast VDAC1 increased the voltage dependence of only one of the two gating processes of the channels. This asymmetrical voltage dependence was the same whether the membrane contained only one channel or hundreds. More importantly, the asymmetry in the voltage dependence varied from one membrane to another, indicating that all the channels in the membrane were in the same orientation, but that this orientation varied from experiment to experiment. Because this variation in the overall orientation could not be accounted for by any experimental asymmetry, the results were explained by proposing that the first channel insertion occurred with a random orientation. Subsequent insertions were directed and catalyzed by the channel already in the membrane. This process is the auto-directed insertion of VDAC channels into phospholipid membranes.

Insertion of VDAC channels into phospholipid membranes probably requires proper orientation of the VDAC protein, a collision with the appropriate site on the membrane, and a collision of sufficient energy to overcome the energy barrier to insertion. The VDAC channels already in the membrane may act as catalysts by interacting with and directing the orientation of the inserting VDAC proteins and reducing the energy barrier to insertion. Direct measurements of the rates of channel insertion (Xu and Colombini, 1996, 1997) support the concept of auto-directed insertion. Indeed the channels in the membrane can be induced to become better insertion catalysts by treatments with urea and guanidinium chloride.

If VDAC channels in membranes act as insertion catalysts, do they catalyze the insertion of other proteins? Do other proteins act as insertion catalysts? In this paper, we have explored these questions by testing the ability of VDAC to catalyze the insertion of a variety of channel-formers: KcsA, PorA/C1 from Neisseria meningitidis, α-hemolysin, and gramicidin and the ability of these proteins to catalyze the insertion of VDAC.

**MATERIALS AND METHODS**

**Brief description of the channel-formers used in this work**

VDAC: A channel responsible for the metabolite permeability of the mitochondrial outer membrane of all eucaryotes. A single 30-kDa polypeptide forms the channel. The walls of the pore are composed of 1 α-helix and 13 β-strands. Its conductance is 0.45 nS in 0.10 M KCl (Colombini et al., 1996; Song and Colombini, 1996).

Gramicidin: This pentadecapeptide from Bacillus brevis forms a β-helix. The channels are head-to-head dimers and selective for monovalent cations. The conductance is 40 pS in 1.0 M KCl (Haas and Hayden, 1970, 1972).

PorA/C1: A highly cation-selective channel formerly believed to form a 16-stranded β-barrel. It forms trimeric channels. The PorA/C1 protein (41 kDa) is produced by N. meningitidis. The conductance of homotrimeric channels is 0.97 nS in 0.20 M NaCl (Song et al., 1996).

α-hemolysin: A water-soluble protein secreted by Staphylococcus aureus. The monomer of α-hemolysin has a mass of 33 kDa. The channel former is a heptamer with a 14-stranded β-barrel transmembrane portion and a highly asymmetrical mushroom-shaped surface domain on one side. The single-channel conductance is about 90 pS in 0.10 M KCl at pH 7.0 (Song et al., 1996).

KcsA: This potassium channel from Streptomyces lividans is composed of four identical subunits. Channel-forming activity can only be observed in acidic solutions. The conductance is 135 pS in symmetrical 0.20 M KCl (Cuello et al., 1998).

**Sources of channel formers**

VDAC proteins were purified from Neurospora crassa mitochondria as previously described (Mannella, 1982; Freitag et al., 1983) and dissolved in 2.5% Triton X100. PorA/C1 porin was a gift from M. S. Blake (Baxter Healthcare Corp., Columbia, MD) as a Zwittergen solution. It was supplemented to 1% Triton X100 to achieve insertion into planar membranes. α-hemolysin and gramicidin were purchased from Sigma Chemical Co. Gramicidin was dissolved in 3:2 dimethyl sulfoxide:dimethylformamide (v/v) and α-hemolysin dissolved in 1.0 M KCl, 1 mM MgCl2 and 5 mM HEPES, pH 7.0. KcsA was a gift from Eduardo Perozo (Univ. of Virginia Health Science Center, Charlottesville, VA) dissolved in 2.5% Triton X100. PorA/C1 porin was a gift from M. S. Blake (Baxter Healthcare Corp., Columbia, MD) as a Zwittergen solution. It was supplemented to 1% Triton X100 to achieve insertion into planar membranes.

As indicated, some channel formers are dissolved in detergents and thus detergent is added to the aqueous solution along with the channel former. The amount added to the chamber was 7 μL into a total of 4.5 to 5 mL. Assuming all the detergent remained in solution, the concentration of detergent present before perfusion would be 0.0035% Triton for VDAC addition, 0.0015% Triton for PorA/C1 addition, and 1.4 μM dodecyl maltoside. After perfusion, residual detergent had no effect on insertion (see Xu and Colombini, 1997).

**Experimental system**

All experiments were performed on proteins reconstituted into planar phospholipid membranes made by the method of Montal and Mueller (1972) as revised by Schein et al. (1976) and Colombini (1987). A phospholipid membrane was formed across a 0.1-mm-diameter hole in a Saran partition using monolayers composed of 5:1 diphytanoyl phosphatidylcholine:cholesterol (w/w). The membrane separated two 5-mL aqueous compartments named cis and trans. The voltage was applied to the cis side and the trans side was maintained at virtual ground by an amplifier in the inverted mode (52K, Analog Devices, Norwood, MA). The KcsA channels were recorded with the Axotape recording system (version 2; Axon Instruments, Foster City, CA). The current was filtered at a frequency of 190 Hz using a Butterworth filter. Calomel electrodes with built-in saturated KCl bridges were used to interface with the aqueous solutions. In the records illustrated, the trans-membrane voltage was −10 mV unless otherwise indicated.

**Statistical tests**

The Student’s t-test was used for assessing statistical significance. The two-tailed test was used and the decision was made at the 95% confidence level. All values are expressed as mean ± SEM (number of experiments).
Experimental strategy

The goal was to assess the ability of a channel former in the membrane (the first protein) to catalyze the insertion of a different channel former (the second protein). Thus, it was necessary to first add and observe the insertion of the first protein and then add the second channel former and assess the ability of the latter to insert into the membrane. Because very few of the added protein molecules actually insert into the membrane, it was necessary to remove most of the excess amount of the first protein before the addition of the second. In addition, it was important to be able to distinguish between the insertions arising from each protein. Finally, it was necessary to minimize spurious factors that might influence protein insertion.

The basic approach involved adding a sample of the first protein to the cis side of a phospholipid membrane at a dose that results in the insertion of one or a few channels. After insertions of this protein were observed, the cis side was perfused with a denser solution to wash out the remaining protein molecules in the solution to stop further insertions and exclude any interactions between the two test proteins in the same aqueous solution (in the occasions when both were added to the same side). In addition, this perfusion removed other solutes, including trace amounts of detergent left in solution.

Perfusions were always performed in the cis side. The perfusing solution was delivered by gravity-driven flow to the bottom of the chamber by means of a narrow-bore tube. A P-10 pipette tip connected to a vacuum system was used to aspirate the surface of the solution to maintain a relatively constant chamber volume. The original solution floated over the denser perfusing solution, allowing complete replacement without the need to stir the solution. At this point, the second sample of protein was added to the trans side, except for the experiments with pre-inserted α-hemolysin, in which VDAC samples were added to either the cis or trans side. By adding the second channel former to the opposite side, we ensured that any interaction must occur via protein (at least one of the two proteins) that spans the membrane as opposed to protein adsorbed to the surface. The amount of the second protein added was initially adjusted to achieve convenient rates of insertion and then kept constant for all subsequent experiments. The final protein concentrations were: VDAC, 0.6 μg/mL; PorA/C1, 0.1 μg/mL; KcsA, 0.2 μg/mL; α-hemolysin, 0.02 μg/mL; and gramicidin, 0.04 μg/mL.

Control experiments were done without the pre-inserted proteins under the same conditions as those used when the pre-inserted channels were present. The amount of added samples and the stirring time while samples were added were kept constant in controls and experiments.

It was critical to be certain that conductance increments attributed to the second protein inserting into the membrane were indeed just that. Despite thorough perfusion, at times, conductance increases due to the first protein still occurred after perfusion. However, such insertions ended in a short time, and the conductance became stable. We believe that proteins absorbed to the membrane surface were most likely the cause of the additional insertions. After the conductance had stabilized, an additional waiting period (typically 5 min) was allowed to further minimize the chances of observing insertions of the first protein after addition of the second. The different properties of the first and second channels, including single-channel conductance, gating properties, and selectivity, were used to confirm that the new insertions were due to the addition of the second protein.

Two parameters were measured for the insertion of the second protein: the delay before the first insertion event, which allowed us to “calculate” the catalyzed insertion rate, and the subsequent “overall” insertion rate. The delay was measured from the end of sample addition to the first insertion event. The overall insertion rate was measured by dividing the total conductance over a certain time by the conductance of a single channel. If the overall insertion rate increased with time, only the initial rate was used.

Experimental conditions

The experimental conditions for testing the catalytic ability of PorA/C1, gramicidin, and α-hemolysin (the trans addition experiment only) on insertion of VDAC were the same. 1.0 M KCl, 1 mM CaCl2, and 5 mM HEPES, pH 7.0 (referred to as “1 M KCl solution”) was initially applied to both sides of the chamber. After a few insertions of the first-added channel former, the cis side was perfused with the same buffer supplemented with 1.0 M sucrose (sucrose/KCl solution).

In experiments investigating whether α-hemolysin facilitates insertion of VDAC added to the same side (the cis addition experiment), the initial solution in the cis side was 0.10 M KCl, 1 mM CaCl2, and 5 mM HEPES (pH 7.0) and that in the trans was the 1.0 M KCl solution. The 1.0 M KCl solution was used as the perfusing solution, thus finally achieving a symmetrical situation (same solutions on both sides) and washing out any leftover α-hemolysin in the solution.

When the catalytic ability of VDAC on insertion of PorA/C1 and α-hemolysin was investigated, both sides initially contained the 1.0 M KCl solution. Because Xu and Colombini (1996) had reported that 5 M urea increases the catalytic ability of VDAC, the cis side was perfused with the 1.0 M KCl solution supplemented with 5.0 M urea after a few VDAC channels inserted into the membrane. The urea also served to increase the density of the displacing solution and denature any uninserted VDAC channels (see Xu and Colombini, 1996).

The experiments testing effects of KcsA on VDAC insertion were performed at low pH because KcsA is activated at low pH (Cuello et al., 1998). The initial solution in the cis side was 1.0 M KCl, 1 mM MgCl2, and 1 mM succinate acid (Na+), pH 4.0. The solution in the trans side was unbuffered 1.0 M sucrose, 1.0 M NaCl and 1 mM MgCl2. We added sucrose in the trans side because we found that the activity of KcsA channels was best observed under such conditions. The different salts allowed us to positively identify the KcsA channels by their selectivity of K+ over Na+...
channels enter the membrane in the absence of any catalyst. The insertion rate calculated from this delay is called the “calculated insertion rate.” The overall rate of insertion of the channels after the first insertion represents a catalyzed insertion rate (as previously described Xu and Colombini, 1996). Sometimes this rate increases as illustrated in Fig. 1 A, and this is interpreted as being due to the presence of new foci of catalyzed insertion (see Discussion). To limit the complexity of the experimental investigation, we limited our studies to the initial portion of the overall insertion rate.

In Fig. 1, B and C, before VDAC addition, other channel formers, PorA/C1, and α-hemolysin, were inserted into the membrane. These were added to one side of the membrane (cis side) and, after a few channels had inserted, the cis compartment was perfused to eliminate excess protein still in solution (see Experimental Conditions for details). The decline in current was due to the presence of sucrose in the perfusing solution. Addition of VDAC to the opposite (trans) side resulted in VDAC insertion but at very different rates in the two cases. The presence of PorA/C1 greatly reduced the delay before first insertion of VDAC and greatly increased the overall rate of VDAC channel insertion. No difference in these parameters was detected with α-hemolysin.

The sample of PorA/C1 added contained 1% (v/v) of the detergent, Triton X-100. However, following perfusion of the cis compartment, the detergent was washed out save perhaps for trace amounts left on surfaces. Xu and Colombini (1997) showed that the presence of Triton on the cis side did not affect the delay before first insertion of VDAC but did increase the subsequent overall insertion rate. However, after perfusion, there was no effect on the overall insertion rate. In these experiments, perfusion of the cis compartment removed the detergent, and, thus, the detergent alone could not have been responsible for the observed acceleration of insertion.

Among the proteins tested in this way, only PorA/C1 and gramicidin reduced the delay before the first insertion of VDAC by a statistically significant extent (Table 1). However, the results with gramicidin were more complex. In Table 1, all the results observed were pooled together, but, in fact, the observations fell into two groups; examples of each are illustrated in Fig. 2. One set of results had very short delays, 13 ± 4 (4) s (Fig. 2 A). The other set had long delays, 227 ± 6 (4) s (Fig. 2 B), and these were not significantly different from the control, 356 ± 90 (6) s. No experimental difference was found that accounted for the two groups. For example, the delay was independent of the number of pre-inserted gramicidin channels.

![FIGURE 1 PorA/C1 accelerates the insertion of VDAC but α-hemolysin does not. (A) VDAC insertion into an unmodified membrane. The VDAC sample was added to the trans side. Individual insertion steps could be clearly distinguished. The delay before the first insertion (double arrowhead) and the initial overall insertion rate were measured. The early insertions defined the overall insertion rate. The rate increased at later times perhaps as a result of the formation of new insertion foci. (B) PorA/C1 accelerates VDAC insertion. PorA/C1 was added to the cis side as indicated. The cis side was perfused with sucrose/KCl solution and this resulted in a conductance drop due to the introduction of 1.0 M sucrose. An aliquot of VDAC was then added to the trans side as indicated. During this portion of the experiment, the chart speed was doubled for a while and then returned to the previous rate (changes in time scale are indicated). The scale of the recording was reduced by four-fold (4×) and then ten-fold (10×) at the times indicated. The delay before the first insertion of VDAC is indicated by the double arrowhead line. The initial process of VDAC insertion has been proportionally expanded in the inset to show the delay time. (C) α-Hemolysin does not accelerate insertion of VDAC channels. The experimental conditions were the same as described in (B). After perfusion, the scale was reduced by five-fold (5×) as indicated. The double arrowhead line represents the delay before the first insertion of VDAC.](image-url)
The delay reflects VDAC’s insertion rate as catalyzed by either PorA/C1 or gramicidin. The subsequent overall rate of VDAC insertion (Table 2) would be the result of the combined catalytic effects of VDAC itself and the other channel formers. PorA/C1 was capable of increasing the subsequent overall insertion rate of VDAC (compare Fig. 1, A and B) indicating that PorA/C1 is a more effective insertion catalyst that VDAC. PorA/C1 was the only protein that was able to do this.

Because α-hemolysin is highly asymmetric, VDAC was added to either the cis (same side as α-hemolysin addition) or the trans side of α-hemolysin-containing membranes.

### TABLE 1 Influence of the presence of a channel former (insertion catalyst) in the membrane on the delay before the first insertion of a newly added channel former (inserting channel)

<table>
<thead>
<tr>
<th>Inserting Channel</th>
<th>Insertion Catalyst</th>
<th>VDAC</th>
<th>PorA/C1</th>
<th>KcsA</th>
<th>α-Hemolysin</th>
<th>Gramicidin</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDAC trans</td>
<td></td>
<td>14 ± 6 (3)</td>
<td>40 ± 8* (9)</td>
<td>180 ± 40 (3)</td>
<td>600 ± 200 (3)</td>
<td>120 ± 40* (8)</td>
</tr>
<tr>
<td></td>
<td>(18-fold)</td>
<td>(9-fold)</td>
<td>(1.4-fold)</td>
<td>(0.6-fold)</td>
<td>(3-fold)</td>
<td></td>
</tr>
<tr>
<td>VDAC cis</td>
<td></td>
<td>15 ± 9 (5)</td>
<td>100 ± 12* (3)</td>
<td>180 ± 90 (3)</td>
<td>440 ± 180 (3)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(9.5-fold)</td>
<td>(4.5-fold)</td>
<td>(1.5-fold)</td>
<td>(1-fold)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PorA/C1</td>
<td></td>
<td>14 ± 6* (3)</td>
<td>40 ± 8* (9)</td>
<td>180 ± 40 (3)</td>
<td>600 ± 200 (3)</td>
<td>120 ± 40* (8)</td>
</tr>
<tr>
<td></td>
<td>(18-fold)</td>
<td>(9-fold)</td>
<td>(1.4-fold)</td>
<td>(0.6-fold)</td>
<td>(3-fold)</td>
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<tr>
<td>KcsA</td>
<td></td>
<td>15 ± 9* (5)</td>
<td>100 ± 12* (3)</td>
<td>180 ± 90 (3)</td>
<td>440 ± 180 (3)</td>
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<td></td>
<td>(9.5-fold)</td>
<td>(4.5-fold)</td>
<td>(1.5-fold)</td>
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</tr>
<tr>
<td>α-hemolysin</td>
<td></td>
<td>14 ± 6* (3)</td>
<td>40 ± 8* (9)</td>
<td>180 ± 40 (3)</td>
<td>600 ± 200 (3)</td>
<td>120 ± 40* (8)</td>
</tr>
<tr>
<td></td>
<td>(18-fold)</td>
<td>(9-fold)</td>
<td>(1.4-fold)</td>
<td>(0.6-fold)</td>
<td>(3-fold)</td>
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</table>

*Statistically significant shortening of delay.
† Xu and Colombini, 1997.

The delay reflects VDAC’s insertion rate as catalyzed by either PorA/C1 or gramicidin. The subsequent overall rate of VDAC insertion (Table 2) would be the result of the combined catalytic effects of VDAC itself and the other channel formers. PorA/C1 was capable of increasing the subsequent overall insertion rate of VDAC (compare Fig. 1, A and B) indicating that PorA/C1 is a more effective insertion catalyst that VDAC. PorA/C1 was the only protein that was able to do this.

Because α-hemolysin is highly asymmetric, VDAC was added to either the cis (same side as α-hemolysin addition) or the trans side of α-hemolysin-containing membranes.

![Figure 2](image-url)  
**FIGURE 2** Gramicidin shortens the delay before the first insertion of VDAC. Experimental conditions were the same as described in Fig. 1 B. Gramicidin still inserted (the smooth increase) after perfusion probably because of its low water solubility. The gramicidin channels had a long open-channel lifetime because the solvent-free membranes used are thinner than the planar membranes used in most published work. The two traces illustrate the short (A) and long (B) delay times observed from VDAC addition to VDAC insertion. The chart speed was doubled as indicated (50-s time bar). The conductance scale was reduced by 10- (10×), 50- (50×), and 200-fold (200×) as labeled.
The insertion of VDAC was not significantly influenced by the presence of \( \alpha \)-hemolysin (Fig. 1 C) in the membrane in either orientation (see the Tables).

A second set of experiments was performed to assess the ability of VDAC to catalyze the insertion of PorA/C1, KcsA, and \( \alpha \)-hemolysin. VDAC in the membrane shortened the delay before the first insertion of both KcsA and PorA/C1 channels. The influence on PorA/C1 was much stronger (Table 1) and probably brings the delay down to the diffusion limit (Xu and Colombini, 1997). VDAC did not significantly increase the overall rate of insertion of PorA/C1 but the catalyzed insertion of PorA/C1 by VDAC that is evident in the reduction of the delay may easily have been masked by the catalysis of the newly inserted PorA/C1.

KcsA channels inserted spontaneously when an aliquot of KcsA dissolved in dodecyl maltoside was added to the aqueous phase (Fig. 3 A). The flickering channels formed by KcsA can be confused with small transients that sometimes appear during the recording. However, at higher time resolution, it was easy to distinguish between transients and KcsA channels. In addition, the use of K\(^+\) and Na\(^+\) salts on opposite sides of the membrane allowed an assessment of the selectivity of the conducting events, ensuring that the events were indeed due to KcsA (Fig. 3 A). Thus, in Fig. 3 B, KcsA was added to the trans side of the chamber in the presence of a single VDAC channel. The first KcsA channel insertion is indicated by a transient upward deflection that is clearly resolved as a single-channel opening event in the high time-resolution record shown just below (Fig. 3 B). So, VDAC did reduce the delay before the first insertion of KcsA, but did not increase the subsequent overall rate of insertion of KcsA channels. This rate was measured as an increase in the open-state probability with time.

**DISCUSSION**

When membrane proteins insert into membranes, they encounter large energy barriers in transferring from the aqueous to the lipid phase. Let us consider two experimental systems. The first consists of membrane proteins released into the cytosol as water-soluble precursors before insertion into the membranes of certain cellular organelles. To remain soluble, the hydrophobic portions of the proteins are likely to be either internalized into the protein core or interacting with chaperones. The second system is that of detergent-solubilized membrane proteins inserting into phospholipid bilayers.

**TABLE 2 Overall rate of insertion of newly added channel former (inserting channel) with specified channel former (insertion catalyst) already in the membrane**

<table>
<thead>
<tr>
<th>Inserting Channel ( \psi )</th>
<th>VDAC ( \psi )</th>
<th>PorA/C1 ( \psi )</th>
<th>KcsA ( \psi )</th>
<th>( \alpha )-Hemolysin ( \psi )</th>
<th>Gramicidin ( \psi )</th>
</tr>
</thead>
<tbody>
<tr>
<td>VDAC ( \psi )</td>
<td>100 ± 20* (3)</td>
<td>400 ± 200† (8)</td>
<td>3.2 ± 1.7 (3)</td>
<td>1.4 ± 0.6 (3)</td>
<td>3.5 ± 0.6 (8)</td>
</tr>
<tr>
<td>PorA/C1 ( \psi )</td>
<td>29 ± 10 (5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>( \alpha )-Hemolysin ( \psi )</td>
<td>12 ± 5 (3)</td>
<td></td>
<td></td>
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</tbody>
</table>

*From Xu and Colombini (1997), taking the highest rate with 5 M urea on the opposite side.
†Statistically significant increase in rate.
membranes. These are stabilized in water by having their hydrophobic regions coated with detergent molecules. Dilution of these into a detergent-free environment to observe insertion into phospholipid membranes must result in loss of some of this detergent coat leaving the protein in a metastable state. In both cases, hydrophobic regions must be exposed to the environment within the membrane and some extensive polar regions must cross to the opposite side of the membrane. The proteins must go through states with rather unfavorable interactions. Clearly this process involves high-energy transitions that could be catalyzed by interactions with appropriate structures. The protein insertion machinery in the endoplasmic reticulum, mitochondria, and other organelles may perform some of this catalysis. In addition, the structure of the inserting protein may have evolved a reaction path that minimizes the formation of high-energy insertion intermediates. However, the fidelity of targeting may be increased and insertion rates may be augmented if proteins already in the membrane catalyze the insertion of appropriate proteins into that membrane. After all, membranes are not generated de novo; rather, existing membranes are expanded followed by some fission event.

Voltage-dependent anion channel (VDAC) channels in membranes have been shown to catalyze the insertion of other VDAC channels into a phospholipid membrane by a process termed auto-directed insertion (Zizi et al., 1995; Xu and Colombini, 1996, 1997). A VDAC channel in the membrane increases the insertion rate of other VDAC channels by 10 orders of magnitude over the rate of insertion into a region of equivalent area of an unmodified phospholipid membrane. Also the inserting channels are induced to insert in only one direction, the same orientation as that of the catalyzing VDAC channel already in the membrane. In this work, we have shown that VDAC can also catalyze the insertion of other proteins. VDAC shortened the time it took for the first KcsA or PorA/C1 channel to appear in the membrane but had no effect on the delay before the first insertion of α-hemolysin.

Rather than being a specific, evolutionarily selected process, one might hypothesize that the observations reported here are purely the result of physical interactions. The mere presence of VDAC in the membrane may disturb the phospholipid structure, resulting in the reduction of the energy barrier to insertion of protein at that site. If so, why was the effect limited to KcsA and PorA/C1 and why was there no effect on the insertion of α-hemolysin? Similarly, of the various channel formers tested, only the presence of PorA/C1 or gramicidin in the membrane had any effect of the insertion of VDAC. Thus, if the disturbance hypothesis is correct, it must be a disturbance that shows quite a high degree of specificity. The distinction between a specific disturbance and the formation of a site for catalyzed insertion is really just semantics.

It must be emphasized that the catalyzed insertion process would have to accelerate the insertion by many orders of magnitude to be detected by the methods described. Thus, negative results should just be interpreted as no detectable effect.

Focusing on the ability of gramicidin to catalyze VDAC insertion, we only observed acceleration of insertion in some of the experiments. In half of the experiments, gramicidin dramatically reduced the delay to first insertion of VDAC (Fig. 2). In the other half, no significant effect was observed. When all the results were pooled (Table 1), the shortening of the average delay was statistically significant, and one could just focus on this average. However, the sharp difference between the two sets of results begs for some explanation. Available evidence strongly indicates that gramicidin channels are head-to-head helical dimers estimated to be 3 nm in length (Finkelstein and Andersen, 1981). So the channel itself is a symmetrical structure. The channel is not expected to extend above the plane of the membrane but rather is believed to cause the lipid surface to dimple somewhat. It is generally believed, although there is no direct evidence, that this dimpling is the same on both membrane surfaces, resulting in a symmetrical structure. However, there is no compelling reason excluding the possibility that the channel may reach the surface on one side of the membrane and form a more prominent dimpling on the other side. Perhaps this asymmetrical structure is of lower energy. If so, then inserted gramicidin channels may form a small patch in the membrane with different properties on the two surfaces, one capable of catalyzing VDAC insertion and the other not. The location of the catalytic surface may simply depend on random chance, thus explaining our results. In half of the experiments, VDAC was added to the compartment facing the catalytic surface of the gramicidin patch. Clearly this is just speculation, and other explanations are possible. However, the possibility that gramicidin monomers adsorbed to the membrane surface are responsible for the accelerated insertion of VDAC, seems unlikely. Recall that VDAC was added to the opposite side of the membrane relative to gramicidin addition, and thus, one would not expect to find gramicidin monomers adsorbed to the membrane surface on that side. Another hypothesis, that a critical number of gramicidin monomers might be required to catalyze the insertion, seems unlikely because the length of the delay did not correlate with the number of gramicidin channels present in the membrane.

Unlike gramicidin, α-hemolysin in the membrane did not reduce the delay to the first insertion of VDAC (Table 1). This is somewhat surprising because α-hemolysin is much larger than gramicidin and has a much more extensive protein–lipid interface. If the nature of the interface were the same, one might expect that α-hemolysin might perturb the phospholipid membrane to a greater extent. When α-hemolysin channels are in membranes, the length of the membrane-spanning stem structure is 6.5 nm (Song et al., 1996). This is longer than the thickness of the membrane (5 nm) and α-hemolysin channels should extend into the aqueous phase on both sides. On the cis side, the crystal structure
shows that α-hemolysin forms a large mushroom-shaped aqueous domain and this may well obscure the lipid-protein interface on that side. However, on the trans side, the aqueous portion is a simple cylinder. Despite the larger area of protein–lipid interface in α-hemolysin as compared to gramicidin, the nature of any lipid disturbance may be critical. With this caveat in mind, the fact that α-hemolysin and KcsA do not reduce the delay before the first insertion of VDAC channels (Table 1) suggests that simple disturbance of the phospholipids is insufficient and that some specificity is necessary to facilitate the insertion process.

A quantitative estimate of the acceleration induced by the catalytic process can be calculated from the delay before the first insertion (Table 3). After the first insertion, the subsequent overall insertion rate may be influenced by autocatalysis. For example, if the delay of VDAC insertion into an unmodified membrane were 350 s, the calculated insertion rate would be 0.2 channels min⁻¹. Normalizing for the membrane area of 1 × 10⁻² mm², the calculated insertion rate per unit area would be 20 channels min⁻¹ mm⁻². If catalysis reduced the delay to 40 s, the calculated insertion rate would be 2 channels min⁻¹. But the catalytic area must be limited by the size of the catalyst (no action at a distance). Taking a high-end estimate of the area of the pre-inserted channel(s) to be 100 nm², the catalyzed insertion rate would be 2 × 10⁻⁹ channels min⁻¹ mm⁻². So the insertion rate calculated from the delay time would have been accelerated by 9 orders of magnitude (Table 3). Clearly, the methods used only allow the detection of large increases in the rate of insertion, and smaller, undetected increases may have occurred with some combinations of proteins.

One might propose that the inserting protein may associate superficially with the membrane diffusing in two-dimensions for some time (“encounter time”: Schwarz, 1987; Wiegel, 1983) before insertion. This would allow the protein to explore a large area of membrane, increasing its likelihood of interacting with the catalyst. During this encounter time, the catalyst is also diffusing laterally, and this too would increase the chances of the two molecules coming into contact. However, the same would be true for any single molecule in the membrane, so these considerations would not affect the calculated acceleration of the insertion rate because it is relative to any other site of equivalent area in the membrane.

There is a clear distinction between reducing the delay to first insertion and accelerating the subsequent overall insertion rate. The former was more frequently achieved and the latter was only observed in one case and accompanied by the former. If one protein were capable of catalyzing insertion as indicated by a reduction in the delay to first insertion and the calculated insertion rate per unit area, why would it not also accelerate the subsequent overall insertion rate? There are at least two possibilities. Perhaps after the catalyzed insertion of the first channel (or the first few) the catalytic site is occupied and unable to function. If so, then the catalytic ability of the first-added protein would be eliminated. Alternatively, the newly-inserted channels might themselves act as catalysts, and their catalytic activity may equal or exceed that of the original channel. The observations with PorA/C1 strongly indicate that it too can catalyze insertion of proteins into membranes. The mutual interaction between PorA/C1 and VDAC is clear. Perhaps the failure of VDAC to accelerate the overall rate of insertion of PorA/C1 indicates that PorA/C1 is a more efficient insertion catalyst for PorA/C1. The fact that PorA/C1 accelerates the overall insertion rate of VDAC shows that PorA/C1 is more effective at catalyzing the insertion of VDAC than is VDAC itself. The ability of other proteins to catalyze the insertion of fellow proteins into phospholipid membranes has been reported (Massari et al., 2000; Song et al., 1999).

If all the proteins being inserted act as catalysts, one might expect an exponential increase in the overall insertion rate. However, what one observes is a fairly constant overall insertion rate sometimes followed by a new higher overall rate that is also rather constant (e.g., Fig. 1 A). This has been interpreted (Xu and Colombini, 1997) as the result of formation of insertion foci. The inserted proteins must first catalyze the insertion of fellow proteins into phospholipid membranes. The catalytic ability of the first-added protein would be eliminated. Alternatively, the newly-inserted channels might themselves act as catalysts, and their catalytic activity may equal or exceed that of the original channel. The observations with PorA/C1 strongly indicate that it too can catalyze insertion of proteins into membranes. The mutual interaction between PorA/C1 and VDAC is clear. Perhaps the failure of VDAC to accelerate the overall rate of insertion of PorA/C1 indicates that PorA/C1 is a more efficient insertion catalyst for PorA/C1. The fact that PorA/C1 accelerates the overall insertion rate of VDAC shows that PorA/C1 is more effective at catalyzing the insertion of VDAC than is VDAC itself. The ability of other proteins to catalyze the insertion of fellow proteins into phospholipid membranes has been reported (Massari et al., 2000; Song et al., 1999).

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overall insertion rate, resulting in a new higher rate of insertion, as is sometimes observed.

The observations of VDAC accelerating the insertion of KcsA hint at the possibility that the inserting KcsA blocked the catalytic site on VDAC. The delay was reduced four-fold but the overall rate of insertion was unaffected. This result was obtained despite the fact that the overall rate of insertion of KcsA was exceedingly slow. Thus one cannot attribute the result to a fast background rate of insertion as was observed with PorA/C1. Often, a single VDAC channel caused the insertion of a single KcsA channel.

The physiological relevance of this catalyzed insertion process is debatable. There are clearly established paradigms for how proteins are targeted to biological membranes, and, therefore, there is a natural skepticism of alternative ideas. The recognition and specificity demonstrated are not easily attributed to VDAC merely disturbing the membrane and creating an interfacial polarity that both catalyzes insertion and biases the insertion direction. The results are consistent with auto-directed insertion being a process that involves specific recognition and alteration of the conformational energy field favoring structures that more easily insert into membranes resulting in specifically folded structures. This process may be an adjunct to the recognized mechanisms and may improve the fidelity of protein targeting. It may be a process that predates the appearance of the recognition/insertion machinery that exists today and has survived because it provides some advantage in facilitating the insertion process.

In summary, the auto-directed insertion mechanism is not very promiscuous, but shows a high degree of specificity. The specificity may lie in a common mechanism of insertion because the proteins that catalyze each other’s insertion most readily also have a very similar overall structure. This specificity also tends to discount a mechanism based on protein-induced membrane disturbance in favor of one that is maintained by natural selection.

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