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Characterization of a Large Conductance, Cation-selective Channel from Sea Urchin Eggs that is Sensitive to Sulfhydryl Reducing Agents

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Abstract. Vesicles containing large conductance cation selective channels were isolated from sea urchin (*Strongylocentrotus purpuratus*) eggs. Addition of the vesicles to one side of lipid bilayer led to the rapid appearance of 200 or more identical channels. These channels would then inactivate within 2 to 10 min. The inactivation could be prevented by the addition of sulfhydryl reducing agents (e.g., dithiothreitol or glutathione) to the *cis* side of the membrane. Only one channel type is present. The channel is cation selective, with a conductance of 572 ps in symmetrical 0.5 M KCl. The relative cation selectivity is K (1.0) > Cs (0.53) ≈ Na (0.52) > Li (0.2). The permeability ratio (P_X/P_K) is 1.37 (Li) > 1.27 (Na) > 0.57 (Cs). Most organic cations (choline, tetraethylamine, tetrabutylamine, gallamine, lysine, histidine, arginine, etc.) and multivalent cations (La^{+3}, alkali earth family, Zn^{+2}, Eu^{+3}, etc.) produced a significant channel block. The highest observed affinity was for La^{+3} which produced a 50% decrease in conductance in 500 mM KCl at a concentration of 8 μM. The biophysical properties of this channel are similar to those of a nonselective channel found in ascidian egg plasma membrane (Dale & DeFelice, 1984). A soluble extract of the egg supernatant can also prevent the inactivation of the channels. Using deactivated channels reconstituted into a planar lipid bilayer as an assay, this factor was partially purified. It is heat and acetone stable with a molecular weight of between 10 and 20 K. One of the major bands remaining in the purest fraction cross reacted with antibodies raised against *E. coli* thioredoxin.

Key words: Sea Urchin egg — Ion Channel — Planar lipid bilayer — Sulfhydryl reagent — Thioredoxin

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Introduction

One of the best studied models of cell regulation is the fertilization reaction in sea urchin eggs. Among the major ionic changes that occur in the oocyte within seconds of the fusion of a sperm to the egg plasma membrane are (i) increase in the permeability of the plasma membrane to Ca^{+2}, Na^+, and K^+ (Eisen et al., 1984); (ii) release of Ca^{+2} from intracellular stores producing an increase in intracellular Ca^{+2} (Epel & Vacquier, 1978; Schmidt et al., 1982; Clapper & Lee, 1985); (iii) alkalization of the egg cytosol by an activation of egg plasma membrane H^+/Na^+ exchange effect (Johnson et al., 1976); and (iv) a change in the redox potential as indicated by increases in NADH/NAD and NADPH/NADP ratio (Whitaker & Steinhardt, 1981). The fertilization of sea urchin eggs also involves a major reorganization of the egg surface coat, reassembled from the vitelline layer, and the fusion of cortical granules with the plasma membrane (Schuel, 1978). It seems probable that some of the ionic changes observed during fertilization result from this fusion of cortical granules with the plasma membrane.

In this paper we describe the properties of an ion channel that is found in an egg vesicle fraction of sea urchin and can be studied by in vitro fusion with lipid bilayers. The biophysical properties of this channel are similar to those of a nonselective channel found in ascidian egg plasma membrane (Dale & DeFelice, 1984). The most interesting aspect of this channel system is that it is very sensitive to sulfhydryl reduction/oxidation reagents. After fusion, the channels usually disappear within a few minutes. This disappearance can be prevented by the addition of reducing agents (e.g., dithiothreitol, glutathione, cysteine, etc.). Concentrations of dithiothreitol (DTT) at as low as 100 nM have some effect. The system is even more sensitive to a soluble egg factor (referred to as the channel ''activator'') that

mimics the activation of DTT and which is active at concentrations corresponding to a 62,000-fold dilution of the concentration present in the egg. Preliminary attempts to identify this factor suggest that it is probably closely related to thioredoxin.

Materials and Methods

Membrane Vesicles Preparation

Sea urchin (*Strongylocentrotus purpuratus*) eggs were disrupted using the procedure described by Clapper & Lee (1985). Briefly, sea urchin were spawned by coelum injection of 500 mM KCl. Eggs were collected in 10°C artificial sea water (ASW: NaCl 460 mM, $MgCl_2$ 27 mM, $MgSO_4$ 28 mM, $CaCl_2$ 10 mM, KCl 10 mM and $NaHCO_3$ 2.5 mM, pH 8.0). The eggs were passed through 125 μ nylon mesh to remove large debris and washed with ASW to eliminate extra potassium. Egg jelly coat was removed by acidifying the medium to pH 5.5 with 1 M HCl. The eggs were then washed twice with zero calcium sea water (0-Ca ASW) with 1 mM EGTA and once with 0-Ca ASW without EGTA. Finally the eggs were washed with homogenization buffer (0.72 M glucose, 1 mM $MgCl_2$, 20 mM Hepes, pH 7.2). All of the following homogenization procedures were carried out in an ice bath. Five ml of packed eggs were homogenized with 20 ml of homogenization medium plus the addition of protease inhibitors: aprotinin (10 μg/ml), leupeptin (10 μg/ml), benzamidin (2.5 mM), soybean trypsine inhibitor (20 μg/ml), and 0.1 mM of EGTA and EDTA. The eggs were gently disrupted using a hand operated glass homogenizer (Wheatman, type A). The cell debris was removed by low speed centrifugation (10,000 × *g*, 20 min) at 5°C. The supernatant (Fraction 1) after the first spin contains both the channel and activator activity. To prepare channel containing membrane vesicles, fraction 1 was then diluted 20-fold with 0.15 M NaCl (20 mM Hepes, 0.1 mM EGTA and EDTA, 1 mM $MgCl_2$, pH 7.2) and centrifuged at high speed (120,000 × *g*, 50 min) at 5°C to pellet the membranous fraction. The channel activity is found in the pellet and the supernatant is discarded. The pellets were then resuspended in 1 ml (for every 5 ml of egg suspension) sucrose buffer (0.5 M sucrose, 1 mM $MgCl_2$, 0.1 mM EGTA and EDTA, 20 mM Hepes, pH 7.2). Vesicles in sucrose buffer were stored in 50 μl aliquots under −70°C. Active vesicles can be stored for up to one year under these conditions.

Purification of the ''Activating'' Factor

Fraction 1 was first precipitated by 50% acetone and the acetone was then removed under a vacuum. The acetone-free supernatant was then heated to 75°C and then centrifuged to remove the precipitate. The supernatant was applied to a Q Sepharose (Pharmacia) FPLC column and eluted at room temperature with a linear 0 to 0.5 M NaCl gradient solution containing 1 M glucose (or urea), 50 mM Tris, pH 7.0. Samples were collected every 2 ml on ice. Fractions with peak activity collected from ion exchange chromatography were concentrated by ultracentrifugation through Centricon YM membranes (Amicon) with 10,000 or 3,000 M.W. cutoff and further purified by application to a size exclusion HPLC column (Bio-Sil SEC-125 column, Bio-Rad). Elution was finished in 20 min at room temperature, with 50 mM Na_2SO_4, 20 mM Na_2HPO_4, and 0.5 M urea, pH 6.8. Fractions were collected every 0.5 ml on ice. The protein concentration of egg extract was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin as a standard or by determination of the absorbance at 280 nm with a spectrophotometer (Beckman) assuming absorbance of 1 is equivalent to 1 mg/ml.

Bioassay of ''Activator'' Activity

Activity of each fraction was assayed by its ability to activate the inactivated channel. The frozen vesicle aliquots are still active, presumably because they contain some residual activating factor. The channels become inactivated by slightly warming the vesicles to room temperature (24–27°C). However, if vesicles were held at this temperature too long, the inactivation is irreversible. To prepare vesicles that could be used for the assay, they were stored at 8°C and carefully warmed by adding small volumes (50–200 μl) of room temperature solution to the vesicles in the 8°C bath. Figure 1, lane 1 shows the criteria used to choose vesicles that were suitable for the assay. Either none or only a couple of channels were detected when the vesicles were squirted on the bilayer when no reducing reagent was in the chamber. But more then 20 channels appeared and persisted when this procedure was repeated after the addition of DTT or egg supernatant to the *cis* chamber (Fig. 1 lane 2 and 3). Protein fractions were assayed by this procedure to determine whether they contain the channel activator.

Thiol Activity Assay

The column fractions were also assayed chemically by using Ellman's method (1959) to quantitate the thiol activity. The assay process is done in a photometer cell to follow the progress of the reduction of DTNB (5,5′-Dithiobis(2-nitrobenzoic acid) by thiol compounds. 10 μl of DTNB (10 mM in 95% ethanol) was added to 1 ml photometer cell containing 200 μl sample solution, 300 μl buffer solution (0.5 M NaCl, 1 M urea, 20 mM Tris, pH 7.4) and 500 μl water. Thiol activity was defined as the absorbance change at 412 μm over a 5-min period.

Immunoblotting

The procedure for detecting thioredoxins by immunoblotting was similar to that of Lim et al. (1988). Proteins were separated by analytical discontinuous native gel electrophoresis. Protein samples were dissolved in 2 M sucrose buffer prior to loading and then loaded on 8% acrylamide stacking gel. The slab gel (10 × 15 cm × 1.5 mm) containing 15% acrylamide was run in 25 mM Tris, 192 mM glycin buffer, pH 8.3 without SDS, at 40 mA until the tracking dye goes to the bottom (about 1 hr). The gel was cooled to 15°C by circulating tap water. The resolved proteins were transferred to nitrocellulose paper by horizontal electrophoresis for 3 hr at 120 V with a water-cooled transfer unit. After blocking with BSA, the paper was incubated over night at 37°C with diluted antibody (1:1000, enzyme activity 515 units/ml; Lim et al., 1988). After washing, the paper was incubated with diluted (1:1000) anti-goat IgG coupled with alkaline phosphatase and visualized by alkaline phosphatase staining.

Single Channel Recordings

The planar lipid bilayers (PLB) were formed in a chamber fabricated out of a flat lucite plate (10 cm × 7 cm by 0.5 cm thick) that was mounted on the stage of a standard microscope. The front chamber was a 7 mm × 7 mm × 2 mm (about 100 μl) deep well in this plate. Lipid solution used was 5% asolectin (Avanti Polar Lipids) in decane (Sigma). The membrane was formed on a 0.2 mm hole drilled vertically in the bottom of this well. The back chamber was formed by drilling a 0.3 mm horizontal channel to meet the vertical hole. The

back electrode was a hollow silver tube inserted in this horizontal channel. This tube was then connected to a Hamilton microliter syringe that provided fine control over the volume of the back chamber. The lipid bilayer was formed by painting the lipid solution on the 0.2 mm hole. The thick membrane should spontaneously thin and form a ring of bulk phase lipid around the margin of the aperture. After thinning, its area could be controlled by adjusting the micrometer syringe connected to the back chamber. The bilayer membrane could be easily observed since it was at the focal point of the microscope. A small volume (1 μl) of the diluted vesicles was squirted directly on the bilayer surface. The insertion of the channels into the bilayer occurred spontaneously. Currents were measured using a standard patch clamp amplifier (Hamill et al., 1981) with a 10-giga-ohm feedback resistor (Eltec Instrument). The unfiltered signal was directly recorded on a strip chart recorder since the current fluctuation steps are much larger then the noise level. The number and size of the single channels was analyzed manually.

Results

A Novel Sulfhydryl Agent-Sensitive Channel

We have incorporated the channels isolated from crude sea urchin egg membrane into solvent-containing phospholipid (5% asolectin) bilayers with the fusion method (Miller & Racker, 1976). Fusion took place spontaneously when small amounts of vesicles were squirted on the planar bilayer. Ca^{+2} or osmotic pressure difference are not required for the incorporation of the vesicles. As described in the methods, when the crude vesicles (without any treatment) are squirted on the bilayer, there would be about 20–200 channels fused into the membrane in 3 min. Over a period of about 10–25 min, the number of active channels gradually decayed to zero. The number of the channels and their duration is variable, depending on the details of the vesicle purification, the temperature that the thawed vesicles are kept at, and the time after thawing. This presumably results from the presence of some ''activating factor'' in the vesicles since egg supernatant could slow the rate of decrease of channel activity in the bilayer (*see* below). We also found that the rate of channel loss can be slowed by the addition of sulfhydryl reducing agent. Treatment with sulfhydryl oxidizing agents, such as DTNB or diamid (1 mM), to membrane vesicles substantially diminished or completely abolished the number of active channels, depending on the duration applied. Oxidation did not affect the single channel conductance. The oxidation effects can be reversed by applying excess amounts of reducing agents either to the membrane vesicles or to the channels on the planer bilayer. Reagents such as, IP_3, ATP, GTP, PKC, and Ca^{+2} had no obvious effect on channel activation.

By carefully controlling the temperature and time, vesicles that had been inactivated but that could still be reactivated by sulfhydryl reducing agents were prepared. Shown in lane 1 of Fig. 1 are the control experiments, with no reducing agents in the *cis* chamber. Usually, only one or two channels were present and they disappeared within a few minutes. Lane 2 of Fig. 1 shows the result of adding increasing concentrations of egg supernatant to the *cis* chamber before the addition of vesicles. At 1/12500 dilution, 7 channels are present at the peak, and they decay in about 2 min. At 1/62000 dilution, the activity is close to or somewhat greater than the control. Lane 3 of Fig. 1 shows the effect of increasing concentrations of DTT. At a concentration of 10 μM, there are 25 channels present and they persist for more than 10 min. Even at concentrations as low as 0.1 μM, there is a definite increase in the number of activated channels.

Purification and Attempts to Identify the Channel Activator

We have been able to partially purify the channel activation factor with a combination of acetone precipitation, heat treatment, ion-exchange chromatography, and gel filtration. The channel activation factor isolated from sea urchin egg has the following characteristics: (i) It has a molecular weight between 10,000–20,000 Dt.; (ii) It is heat (85°C) and acetone (50%) stable; (III) One of the proteins of this fraction can cross react with *E. coli* thioredoxin antibody. A summary of the purification procedure is listed in Table 1. The purification steps were complicated by the tendency of the sea urchin egg protein to self aggregate. The aggregated proteins passed through most columns in the void volume and lost all of the activities. The presence of 1 M urea or glucose in all elution solutions tended to reduce this aggregation. Thiol molecules are easily oxidized in the air, therefore we always incubate sample fraction with 2 mM DTT under nitrogen for 30 min prior to each fractionation step.

Pretreatment

The first purification step of the egg extract was 50% acetone precipitation. This supernatant was then heated to 75°C in boiling water for 10 min and the activity remained in the supernatant.

Ion Exchange Chromatography

The heat and acetone precipitated supernatant was fractionated on a Q Sepharose ion exchange column. Fractions collected were first analyzed for thiol concentration by Ellman's method (1959) and those containing thiol activity were assayed for ''activator'' activity using the vesicles described above. Figure 2 is the elution profile at O.D. 280 (unbroken line) with the inserts showing the vesicle assay at selected regions. The thiol activity (ΔA412/5 min) is illustrated in filled circles. There were

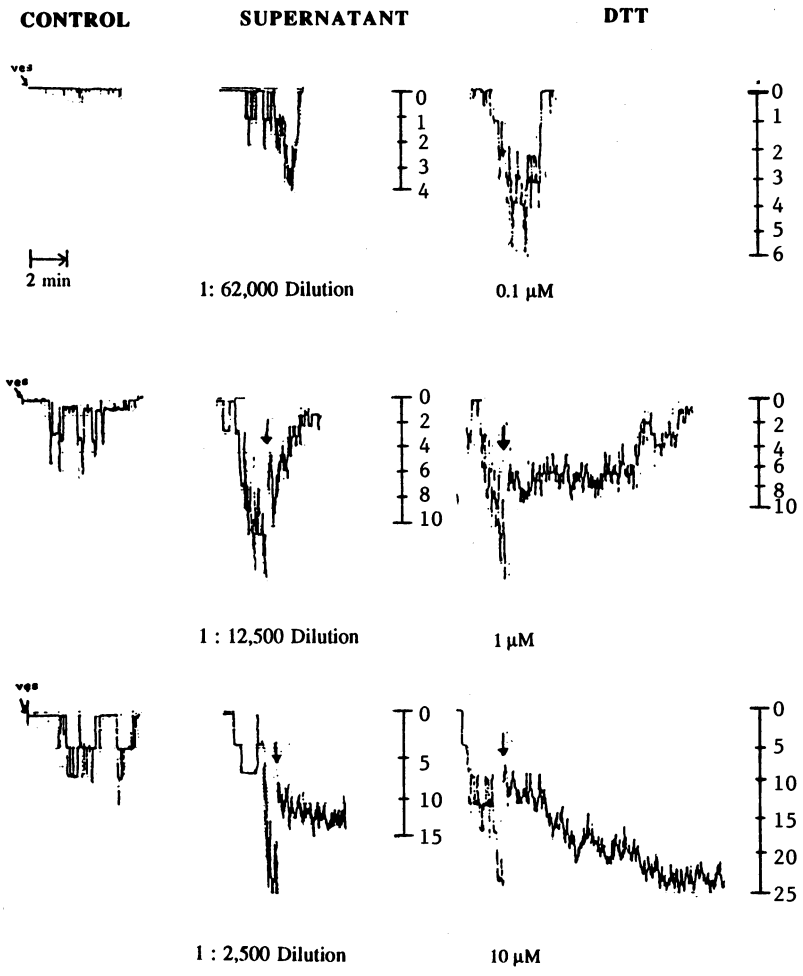

Fig. 1. Appearance of channels after 1:160 vesicle dilution is squirted on membrane. Arrows indicate gain change after numbers of fused channels increase. Scales showing number of open channels is for after the gain change. From left to right: lane 1 — controls, with no addition of any reducing reagent in the bath; lane 2 — dilutions of sea urchin egg supernatant was added before vesicles; lane 3 — dithiothreitol (DTT) added to bath before vesicles.

Table 1. Purification of channel activation factor from 8 ml of sea urchin egg (*Strongylotus purporatus*) suspension (1 million eggs/ml)

Purification step	Volume (ml)	Protein conc. (mg/ml)	Total protein (mg)	Total activity (units)	Specific activity (unit/mg)
Egg supernatant	40	3	120		
Acetone treatment	30	1.3	39		
Heat treatment	28	1.25	27		
Ion exchange	18	1.238	22.28	17.23	0.773
TSK-125 Gel filtration	17	0.089	1.51	7.769	5.14

Protein assay before ion exchange was measured by Bradford's method. After ion exchange, proteins were estimated by absorbance $A_{280} = 1$ is taken as equivalent to 1 mg/ml protein. The activities were measured in a total volume of 1,000 μl sample solution using 10 μl of 10 mM DTNB as described under material and method (enzyme assay). One unit of activity corresponds to $\Delta A_{412}/\text{min} = 1$; specific activity is given as units/mg of protein.

three major peaks showing thiol activity in this elution profile. It can be seen that the highest thiol activity is in the first peak, however, this peak has only weak ''activator'' activity. There were about 12 channels opened and these channels rapidly decayed. Peak 2 can activate more channels than peak 1 but the activity also decayed within 2 min. The highest activator activity is found in the last peak (peak 3), which corresponds to fractions collected at 39–42 min and has relatively low thiol activity. For this last peak, more than 25 channels opened simultaneously and remained for more than five min. Several small sulfhydryl reducing agents are present in sea urchin eggs. Glutathione is present in high concentration (2 mM) in its reduced form as is cysteine, although, in somewhat lower concentrations (Turner et al., 1986). When glutathione and cysteine were applied to

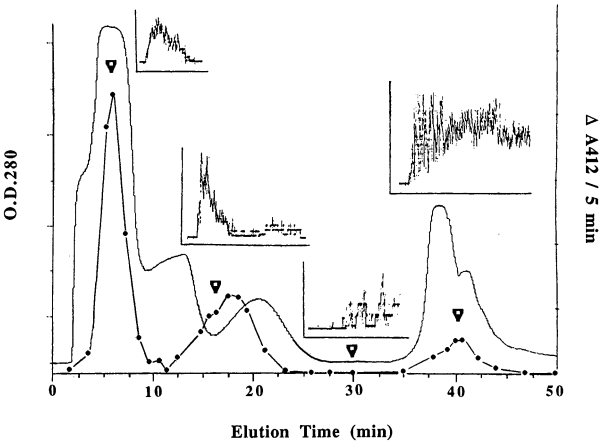

Fig. 2. Ion exchange chromatography of 10 ml pretreated (acetone and heat treated) egg extract through Q Sepharose (anion exchanger) column. Elution was accomplished at room temperature with a flow rate of 2 ml/min. Gradient was run first with a 25-min wash of buffer A (1 M glucose, 20 mM Tris buffer, pH 7.4), then a 15-min linear NaCl gradient from 0 to 0.5 M of NaCl in buffer A, then another 10-min elution of 0.5 M NaCl in buffer A. Unbroken line shows UV absorbance at 280 n. Filled circle (●—●) indicates thiol activity calculated by Ellman's method, measured by UV absorbance at 412 nm over a period of 5 min. Inserts of channel activity represent the biological assay of eluted samples collected at 6 min, 17 min, 30 min, 40 min, respectively (heavy arrows). Sample collected at 40 min had the highest concentration of activating factor.

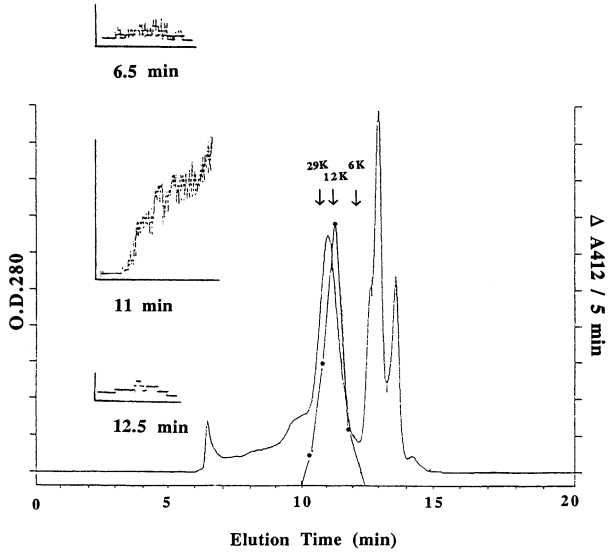

Fig. 3. Gel filtration chromatography of the active fraction eluted after ion exchange column. Elution was accomplished at room temperature with a flow rate of 1 ml/min. Eluent used was 0.5 M Urea, 50 mM Na_2SO_4, 20 mM Na_2HPO_4, at pH 6.8. Fractions were collected every 0.5 ml on ice. Unbroken (—) line shows UV absorbance at 280 nm. Unit thiol activity of each 0.5 ml sample was calculated as ΔA412/5 min (●—●). Standard proteins for calibration are Carbonic anhydrase (29 K, 10 μg), thioredoxin (*Spirulina platensis*, 12K, 10 μg), and insulin (6 K, 5 μg), each peaked at 10.71, 11.17 and 12.16 min respectively. The active fraction has a peak elution time between 11 to 11.5 min, corresponding to a molecular weight between 12 to 20 K. Inserts of channel activity represent the biological assay of eluted samples collected at 6.5 min, 11 min, and 12.5 min, respectively.

the Q Sepharose column, they did not bind to the column and came off in the area that corresponds to the first peak in Fig. 2. *E. coli* thioredoxin has an elution profile similar to that of the activation factor at peak 3 (*data not shown*).

Size Exclusion Chromatography

The active fractions from peak 3 were further concentrated by ultracentrifugation with a 3,000 cutoff filter and applied to a Bio-Sil SEC-125 column. In this case there was only one peak (Fig. 3, elution time between 11 and 11.5 min) with thiol activity and this peak coincided with the peak of activator activity. Calibration of the column using protein molecular weight standards indicates the activity had a molecular weight in the range of 12 to 20 K. This is consistent with results of centrifugation through YM Centricon membranes of varying size. More than 90% of the activity was retained on a 3,000 and 80% on a 10,000 cutoff filter, while only a small fraction of the activity was retained by the 30,000 cutoff filter.

Electrophoresis and Western Immunoblot

Two identical sets of samples were electrophoresed on native gels. One was transferred to nitrocellulose paper for immunoblotting, and the other was stained by Coomassie blue (Fig. 4). Lane 1 was loaded with 2.4 μg *E. coli* thioredoxin as a standard reference. Lane 2 was loaded with acetone extract of sea urchin egg homogenate. Lane 3 was loaded with the active fraction (Fraction 11 collected at 11–11.5 min of Fig. 3) from the size exclusion column. Lane 4 was loaded with fraction collected immediately after the active fraction at 11.5–12 min. Lanes 5, 6, 7 were loaded with twice the amount of that of lanes 2, 3, and 4. On the native gel, Fraction 11 consisted of four major bands and two minor bands (Fig. 4*a*, lane 3 and 6). One of the major bands ran close to that of standard thioredoxin. This band was also significant in the acetone extract (lane 2 and 5). In the immunoblot, only one band cross reacted with the polyclonal antibody raised against *E. coli* thioredoxin. This band coincided with the major band that had the same mobility as the thioredoxin.

Biophysical Characterization of Single Channels

Single Channel Conductance and Gating Behavior

All of the single channel studies were carried out with the addition of 10 μl of 10 μM DTT in the *cis* chamber so that

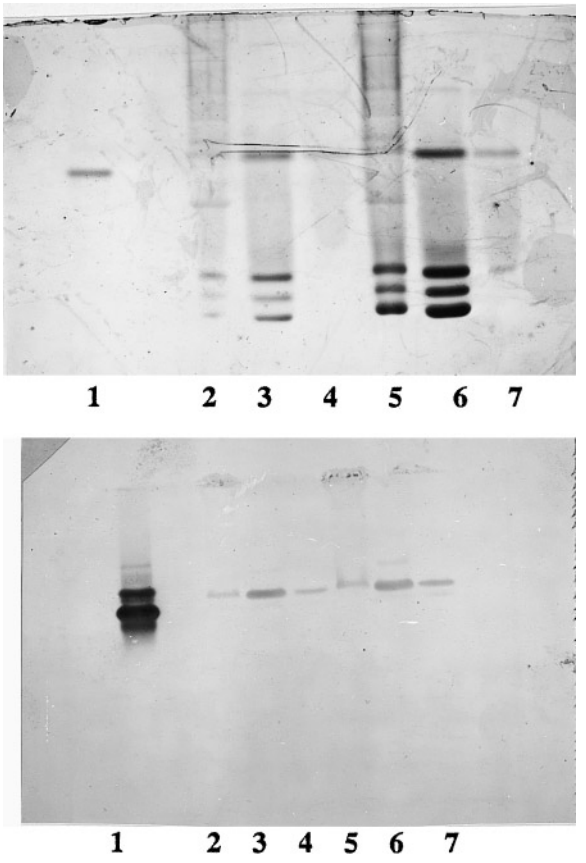

Fig. 4. Electrophoresis and Western Immunoblot of the channel activation fraction. (*a*) Coomassie blue stained native gel of the activation factors and sea urchin egg extract. Lane 1, loaded with standard reference protein, 2.4 μg *E. coli* thioredoxin. Lane 2, acetone extract of egg homogenate. Lane 3, gel filtration fraction collected between 11.0–11.5 minute. Lane 4, gel filtration fraction collected between 11.5–12.0 minute. Lanes 5, 6, and 7 loaded with 2× of the sample of lanes 2, 3, and 4. (*b*) Immunoblotting pattern of sea urchin egg extracts on nitrocellulose paper visualized by alkalin phosphatase. Polyclonal antibody was raised against *E. coli* thioredoxin. Samples were loaded the same way as those in (*a*). Multiple bands shown in standard sample of lane 1 was due to the degraded residue of *E. coli* thioredoxin. Samples from sea urchin egg extract have a band that cross reacts with *E. coli* thioredoxin antibody. The degree of cross reactivity was in proportion to the amount of the sample loaded.

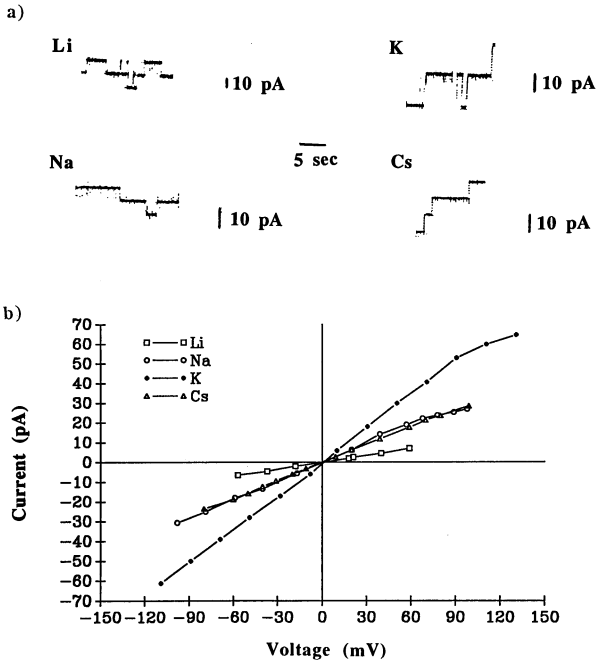

Fig. 5. (*a*) 10 μM Dithiothreitol activated single channel recordings at +30 m V in symmetric 0.5 M alkali chloride solution with 20 mM Hepes, 0.1 mM EGTA & EDTA, pH 7.2. The typical single channel fluctuation shows well-defined unitary conductance levels and the rather long open life time of the channel. (*b*) Single channel *I–V* relationships obtained in 0.5 M symmetric solutions at room temperature. Each data point was averaged from at least two measurements. Fewer data points were collected for Li due to the unstable gating behavior of channels in Li solution. (△) Cs, (●) K, (○) Na, (□) Li.

the channel remained activated. The vesicle solution was diluted 200× before it was squirted onto the bilayer. Too much dilution (over ×1,000) may cause irreversible inactivation of the channels. The number of channels can be reduced by adding lipid to the membrane and removing the excess lipid until there were only two or three channels in the membrane. The single channel current traces for Li^+, Na^+, K^+, and Cs^+ in symmetrical 0.5 M solution (20 mM Hepes, 0.1 mM EGTA & EDTA, 10 μM DTT, pH 7.2) at +30 mV are shown in Fig. 5*a* and the conductance measurements of Fig. 5*a* are tabulated in Table 2. The potassium conductance of 570 ps is among the largest reported for any cation channel. The current-voltage relation for the four cations is shown in Fig. 5*b*. The curves are approximately linear over the range of −100 to +100 mV for alkaline metals. The conductance at +30 mV *vs.* concentration is shown in Fig. 6. For all the cations, the conductance nearly saturated at concentrations of about 0.5 M. Measurements of channel conductance in solutions with ionic strength lower than 200 mM was difficult because the channels disappeared at low ionic strength. This might be because fusion had an ionic dependence or the channel had to stay in high salt solution to remain stable. Since all electrolytes (organic and inorganic) altered the channel conductance, we could not add an ''inert'' electrolyte to maintain the ionic strength while the concentration of the cation of interest was lowered. Lowering the temperature to 18°C allowed us to measure a few channels at concentrations as low as 70 mM and this procedure was carried out only for K^+. For the other cations in Fig. 6, 200 mM was the lowest measurable concentration.

It is difficult to quantitate the voltage dependence of the gating in our membrane preparation. The number of channels first increases and then slowly decays, even with high concentrations of DTT. However, the qualita-

Table 2. Reversal potential, permeability ratio, and conductance of alkaline metals, T = 21–28 °C

Ion	0.2 M		1 M		Conductance (ps) in
X	E_{rev} (mV)	P_x/P_k	E_{rev} (mV)	P_x/P_k	0.5 M
Li	8.2 ± 0.3	1.37 ± 0.02 (2)	45.5 ± 1.5	5.89 ± 0.35 (2)	114 ± 3 (n = 2)
Na	6.0 ± 0.5	1.26 ± 0.02 (2)	7.1 ± 0.6	1.32 ± 0.03 (2)	291 ± 9 (n = 3)
K	0.0	1.00	0.0	1.00	572 ± 25 (n = 16)
Cs	−14	0.57 (1)	−17.0	0.53 (1)	303 ± 11 (n = 2)

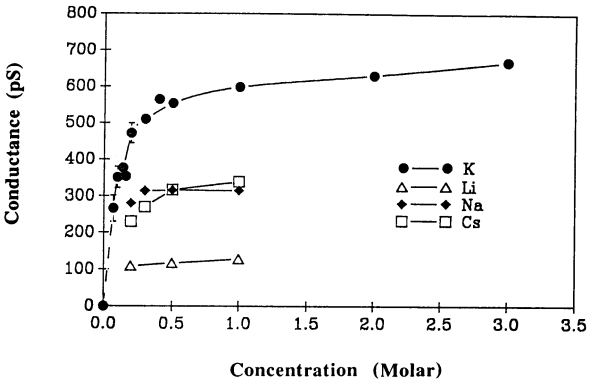

Fig. 6. Single channel conductance *vs.* concentration curves measured at +30 m V for symmetric solutions of (●) KCl, (△) LiCl, (♦) NaCl, and (□) CsCl. The curve for KCl was extrapolated to 0 M from 0.07 M. Each point represents the average of at least two measurements.

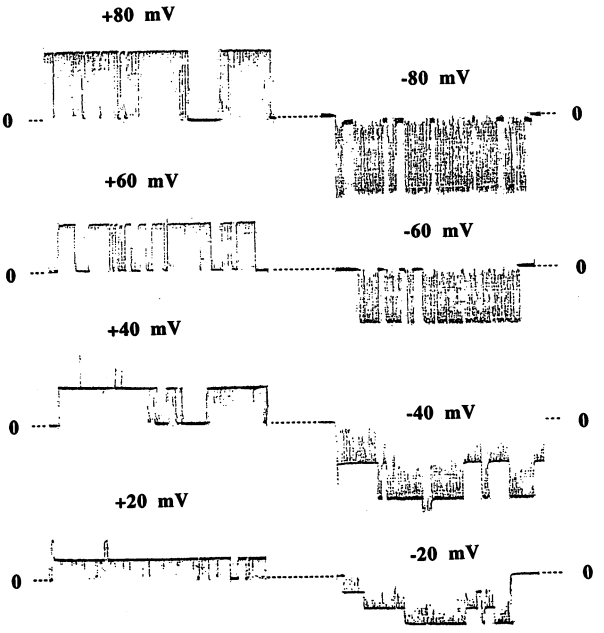

Fig. 7. Voltage-dependent gating behavior of DTT-activated channels on planar bilayer. Positive current is defined as the current from the *cis* side to the *trans* side of the membrane. The broken lines of each trace mark zero current, and upward current trace represents positive current. Records are collected from different preparations. Channels were recorded in 0.5 M KCl, with 20 mM Hepes, 0.1 mM EGTA & EDTA, pH 7.2. The open probability decreases at large negative voltages.

tive dependence of gating on voltage is apparent in Fig. 7. At high negative voltages the channel tends to spend more time in the closed state and has frequent ''flickering closures'' that are not well resolved using our bilayer preparation. At a voltage of −100 mV (*not shown*) the channel was closed for more than 95% of the time, and all the openings were only brief ''flickers.''

Permeability Selectivity

The permeability ratio (P_x/P_y) was determined from the reversal potential (E_{rev}) when ion *X* was on the *cis* side and ion *Y* was on the trans side at activities a_x and a_y, respectively (Hille, 1984):

$$P_x/P_y = (a_y/a_x)\, e^{-(FE_{rev}/RT)}$$

The reversal potentials and the permeability ratios for Na^+, Li^+, and Cs^+ *vs.* K^+ at either 200 mM or 1 M are listed in Table 2. The permeability sequences follow the order of $P_{Li} > P_{Na} > P_K > P_{Cs}$, which decreases monotonically with increasing ion size. The most interesting results are for Li^+. The permeability ratio of Li to K has a strong dependence on concentration which indicates that there must be multiple cation binding sites in the channel (Hille & Schwarz, 1978; Levitt, 1986).

Cation Block of Channel Conductance

All the organic and multivalent cations that were tested produced a block of the K^+ conductance. This included choline, TEA, TBA, Tris, Quinidine, Gallamine, arginine, alkali earth family, Mn^{+2}, Zn^{+2}, Eu^{+3}. The 50% block was estimated by interpolating to the concentration that produced a 50% block of the 500 mM K^+ conductance at +30 mV in symmetrical solutions. These estimated values were Mg (2 ~ 3 mM), Ca (1 ~ 2 mM), Sr (0.2 ~ 0.3 mM), Ba (0.1 ~ 0.2 mM), TEA (30 mM), choline (20 mM), etc. The cation that had the highest affinity was La^{+3} (8 μM). As would be predicted for such a high affinity blocker, a flickering block of the channel could be resolved in our bilayer membranes (Fig. 8*a*). The

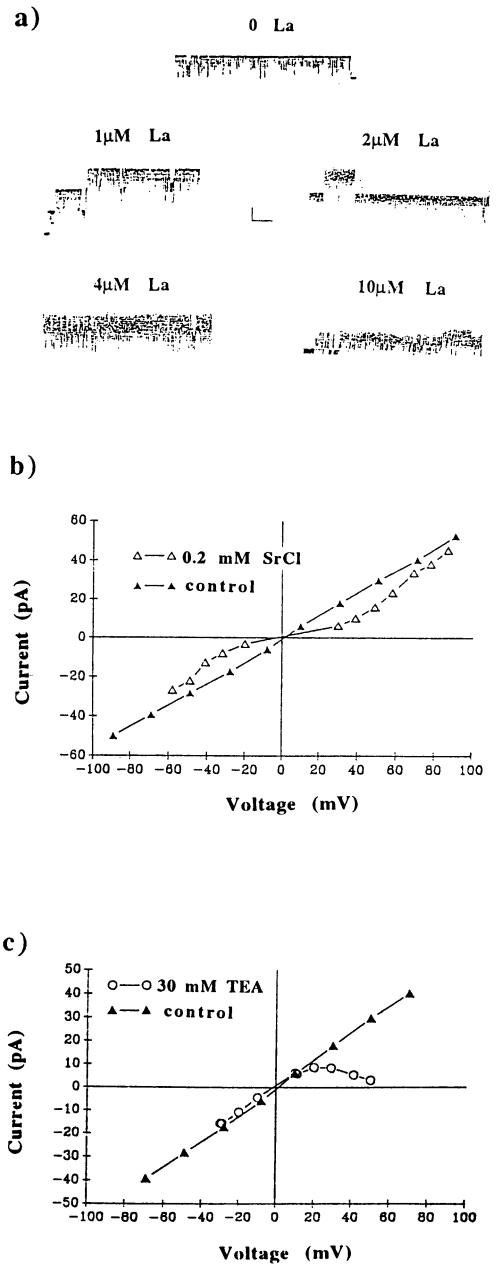

Fig. 8. (*a*) Effect of lanthanum concentration on single channel conductance. One to three single channels were incorporated in the planar bilayer in symmetric 0.5 M KCl, 20 mM Hepes, pH 7.0. Records are collected from different preparations and measured at +30 mV. Scale indicates 10 p A *vs.* 4 sec. La^{+3} concentration is estimated by assuming all the added La^{+3} is free. Records show channel behavior in 0 $LaCl_3$ (control), 1 μM $LaCl_3$, 2 μM $LaCl_3$, 4 μM $LaCl_3$, and 10 μM $LaCl_3$. With the addition of 1–2 μM La, the channel flickering increases. At 4 μM La, the closing intervals were too short to be resolved by our amplifier. With the addition of 10 μM La, the apparent ''single channel conductance'' has significantly decreased. The 50% block concentration of La is about 8 μM. (*b*) *I–V* relations showing voltage dependent block by divalent cations of potassium current. Solutions contain 0.5 M KCl and, (▲) control—0.1 mM EGTA & EDTA; (△)—0.2 mM $SrCl_2$. The block is more significant at low voltages. Larger voltages, both positive and negative, release the block. Similar results were obtained from solutions of $CaCl_2$ or $MgCl_2$. (*c*) *I–V* relation of a voltage dependent block by large organic cations of potassium current. (*o*) Symmetric solution of 30 mM TEA and 0.5 mM KCl (0.1 mM EGTA & EDTA, pH 7.2). (▲) Control, symmetric 0.5 M KCl without TEA. At voltages greater than +20 m V, the channel becomes blocked. No block was observed at negative voltages.

flickering block was also observed for Ba and organic cations, while for Mg, Ca, Sr no such flickering could be detected.

For the divalent cations, the block was greatest at low voltage and tended to be relieved at high voltages and was approximately symmetrical. Figure 8*b* illustrated this for Sr^{+2}. Similar results were obtained with Ca and Mg. In contrast, for large organic cations, such as TEA, TBA, gallamine, and choline etc., the block was highly asymmetrical. There was almost no block from the trans side (negative potentials) and the block was increased at large positive voltages (Fig. 8*c*).

Anions

The anion permeability was estimated from measurements of the reversal potential with 1 M KCl on the trans side and 1 M TEACl on the *cis* side. The reversal potential was approximately +98 mV which would correspond to P_{Cl}/P_K of 0.02 if TEA were impermeable. If TEA were permeable, this ratio would be even smaller. In any case, the channel seems to be highly cation selective.

Discussion

The experiments described here demonstrate that there is a vesicle fraction in sea urchin (*Strongylocentrotus purpuratus*) eggs that contains a reservoir of identical high conductance (570 ps in symmetrical 0.5 M KCl), cation selective ion channels. The injection of 1 μl of a fresh vesicle preparation results in the incorporation of up to 200 channels in the bilayer. This 1 μl should be equivalent to about 1 egg. Thus there must be a minimum of about 200 channels per egg. This is probably an underestimate if one takes into account oxydization and loss of channel activity during purification.

The properties of the channel are similar to those described by Dale & DeFelice (1984). Using patch clamp experiments on the plasma membrane of an ascidian egg, Dale & DeFelice observed a large conductance (400 ps in sea water), nonselective channel whose appearance was synchronous with the change in membrane potential during fertilization. Fertilization of both

sea urchin and ascidian eggs induces an increase in inward Na^+ current that has the following properties (Kozuka & Takahashi, 1982; DeFelice & Kell, 1987; David et al., 1988; Lynn et al., 1988): (i) The current results from a relatively nonselective increase in cation permeability; (ii) Hyperpolarization decreases the open channel probability; (iii) The current cannot be induced in unfertilized eggs simply by changing membrane potential, suggesting that there must be a biochemical step in their activation. All of these observations are consistent with the properties of the channels described in this paper.

These channels are sensitive to sulfhydryl oxidation/reduction. In the absence of reducing reagent in the *cis* chamber, the channels first appear and then rapidly disappear. This disappearance is prevented if reducing reagent is present in the *cis* chamber, suggesting that oxidation/reduction acts primarily on channels after they have been inserted in the membrane and it has little or no effect on the channel insertion process. Usually the DTT or the activating factor is added to the *cis* chamber before the vesicles are added. Once the channels have become inactivated for a few minutes, they cannot be reactivated, suggesting that whatever change that occurs when they are oxidized is irreversible.

Is the Activating Factor Thioredoxin?

The channels are exquisitely sensitive to the egg supernatant activating factor. Dilutions of the normal egg concentration by up to 62,000 are still active. This factor has several similarities with thioredoxin. Thioredoxin is a small, ubiquitous heat-resistant protein that can reduce disulfides at a rate many times faster than DTT (Holmgren, 1985, 1989). Thioredoxin and the supernatant factor have similar molecular weights, are heat stable and have similar elution profiles on ion exchange and size exclusion columns. Most importantly, one of the four major bands in the active fraction cross reacts with antibody raised against *E. coli* thioredoxin. However this factor cannot be identical to thioredoxin because there was no increase in channel activity when mixtures of bacteria thioredoxin, thioredoxin reductase, and NADPH were either added to the *cis* chamber or incubated with the vesicles. Thioredoxin from two different bacterial systems (*E. coli* and *C. Nephridii*) were tried. This thioredoxin rapidly reduced the disulfide groups in the standard insulin assay (Holmgren, 1984) indicating that it was in the reduced form and should have acted on the channel system. In addition, an attempt was made to oxidize (and therefore inactivate) the activating factor by incubating it with thioredoxin reductase plus an excess of NADP. This incubation had no effect on the activating factor. Finally, the activating factor has a strong tendency to self aggregation and it rapidly inactivates in dilute solutions, properties which are not observed for *E. coli* thioredoxin.

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