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Reconstitution in Planar Lipid Bilayers of a Voltage-Dependent Anion-Selective Channel Obtained from Paramecium Mitochondria

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Summary. We have incorporated into planar lipid bilayer membranes a voltage-dependent, anion-selective channel (VDAC) obtained from *Paramecium aurelia*. VDAC-containing membranes have the following properties: (1) The steady-state conductance of a many-channel membrane is maximal when the transmembrane potential is zero and decreases as a steep function of both positive and negative voltage. (2) The fraction of time that an individual channel stays open is strongly voltage dependent in a manner that parallels the voltage dependence of a many-channel membrane. (3) The conductance of the open channel is about 500 pmho in 0.1 to 1.0 M salt solutions and is ohmic. (4) The channel is about 7 times more permeable to Cl^- than to K^+ and is impermeable to Ca^{++}. The procedure for obtaining VDAC and the properties of the channel are highly reproducible.

VDAC activity was found, upon fractionation of the paramecium membranes, to come from the mitochondria. We note that the published data on mitochondrial Cl^- permeability suggest that there may indeed be a voltage-dependent Cl^- permeability in mitochondria.

The method of incorporating VDAC into planar lipid bilayers may be generally useful for reconstituting biological transport systems in these membranes.

Since many biological transport systems have electrical manifestations (conductance, potential, etc.), planar lipid bilayers, which separate two essentially infinite compartments and allow control of both the solution compositions on the two sides of the membrane and the potential difference across the membrane, are particularly suitable for studying their reconstitution. The ability to easily and rapidly control the membrane potential is particularly critical for the study of reconstituted voltage-dependent conductance pathways such as those from nerve and muscle. Several transport systems have been reconstituted in small lipid vesicles (e.g., Hinkle, Kim & Racker, 1972; Goldin & Tong, 1974; Hilden, Rhee & Hokin, 1974; Racker & Stoeckenius, 1974; Knowles & Racker, 1975); although some success has also been claimed with planar bilayers (e.g.,

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Montal, 1974*b*; Semenza, 1974), to date, the molecules studied in these bilayers have mostly been antibiotics (e.g., valinomycin, nystatin, and monazomycin) whose activity depends on their ability to enter membranes from an aqueous phase.

One approach to incorporating intrinsic membrane proteins into artificial membranes is to add them to the membrane-forming solution – either a decane-lipid mixture used to "paint" black lipid membranes (Mueller, Rudin, Tien & Wescott, 1963) or a hexane-lipid mixture used to make hydrocarbon-free planar bilayers (Montal, 1974*a*). In the latter method, lipoprotein monolayers are formed at an air-water interface, and two monolayers are then brought together to form a bilayer. A major difficulty is in properly dispersing the biological membrane material in hydrocarbon. The novelty of our approach is in the method of achieving this dispersion. Biological membrane preparations are sonicated with a 25-fold excess of phospholipid (asolectin), and then lyophilized and suspended in hexane. (By this means we hoped to isolate each protein molecule in a large amount of lipid.) While attempting in this way to reconstitute the voltage-dependent calcium channel of *Paramecium aurelia*, we succeeded in incorporating into planar bilayers a voltage-dependent anion-selective channel (VDAC); we localized this activity to mitochondria.

Materials and Methods

Cell Strains and Cell Growth

P. aurelia (syngen 4, stock 51-s) was grown in a cerophyl medium (Schein, 1976[1]) which had been inoculated with *Aerobacter aerogenes*. *P. aurelia* (syngen 8, stock 229-s), a gift of Dr. A.T. Soldo, was grown both in crude axenic medium, following exactly Soldo, Godoy, and van Wagtendonk (1966) and in defined medium, following exactly Soldo and van Wagtendonk (1969).

Mixed Membrane Fraction

One to two liters of stationary-phase cells were harvested and washed with Dryl's salt solution (Dryl, 1959) (in mM: 2 Na-citrate, 1.5 $CaCl_2$, 1 NaH_2PO_4, 1 Na_2HPO_4). The 0.5 to 1.0 ml of packed cells were resuspended in 18 ml Dryl's solution, 2 ml 95% ethanol added, and the suspension left on ice with infrequent shaking for ten min. (The ethanol treatment is a modification of Watson and Hopkins (1962) procedure for deciliation.) The suspension was spun at $500 \times g$ for 3 min. (When desired, the supernatant was spun at $10{,}000 \times g$ for 10 min to pellet cilia.) The pellet of cell bodies was resuspended in from 5 to 10 ml cold Dryl's

1 Schein, S.J. 1976. A nonbehavioral selection for pawns, mutants of *Paramecium aurelia* with decreased excitability: Genetic and phenotypic characterization (*in preparation*).

solution and twice forced through a 1/2 inch 26 gauge needle at a rate of 1 ml/5 sec. This suspension of broken cell bodies was layered, 5 ml/tube, on a 32 ml discontinuous sucrose gradient (16 ml 50% sucrose, 16 ml 40% sucrose, both buffered with 10 mM Na-HEPES, pH 7.7) and spun in an SW27 rotor at 25,000 rpm for 2 hr. The 40–50% sucrose interfacial material (mixed membrane fraction) was recovered and washed three times with ice-cold 1 mM KCl, 1 mM Tris-HCl (pH 7.5) by pelleting at $10{,}000 \times g$ for 10 min.

Preparation of Purified Pellicles

Paramecium pellicles were purified by a modification of the method of Hufnagel (1967). 9 volumes of cold phosphate-buffered (10 mM, pH 6.9) raffinose (200 mM) were added to 1 volume packed paramecia. The suspension was homogenized by passage through a 1/2 inch 26 gauge needle at a rate of 1 ml/5 sec, five times, centrifuged at $2000 \times g$ for 2 min, and the pellet (of pellicles) washed three times with ice-cold 1 mM KCl, 1 mM Tris-HCl (pH 7.5) by pelleting at $5000 \times g$ for 5 min.

Fractionation of Pellicle-Free Supernatant and Preparation of Mitochondrial-Rich Fraction

The supernatant of the above 2 min $2000 \times g$ spin was layered, 5 ml/tube, on a step gradient of sucrose (4.5 ml each of 60, 55, 50, 45, 40, 35, 30% sucrose, all buffered with 10 mM Na-HEPES, pH 7.7) and spun in an SW27 rotor at 25,000 rpm for 4 hr. Fractions (including the pellet) were recovered from above (fraction 40–45 is all of the 40% fraction down to and including the interface between the 40 and 45% sucrose layers, etc.) and washed three times with ice-cold 1 mM KCl, 1 mM Tris-HCl (pH 7.5) by pelleting at $10{,}000 \times g$ for 10 min.

Treatment of Test Material for Dispersion in Hexane

To a sample of membranes suspended in 1 mM KCl, 1 mM Tris-HCl (pH 7.5), asolectin was added so as to produce a final asolectin to protein ratio of 25 : 1 (w : w); the volume was adjusted so that the final asolectin concentration was 1–2% (w/v). This mixture was sonicated under N_2 at 0 °C in the Cup Horn attachment to the Branson Sonifier (model W185) at setting # 10 (140 W) for 10 min, lyophilized, and stored dessicated at −20 °C. A 1.4% (w/v) suspension in hexane of the lyophilized powder was usually used to make membranes. Often, the hexane suspension was itself sonicated under N_2 as above for 5 min. Both the lyophilized powder and the hexane suspension were stable for at least several months at −20 °C.

Formation of Bilayers and Electrical Measurements

Membranes were made by the method of Montal (1974*a*) across a 0.1 to 0.2 mm diameter hole (made with a hot stainless steel wire) in a Saran Wrap partition separating two Teflon compartments (called "front" and "rear"), each of about 7 ml capacity and 6.5 cm^2 surface area (Fig. 1). Before starting an experiment, both the partition and the compartments were washed with 2 : 1 chloroform : methanol. The partition was then coated with vaseline, using a Q-tip dipped in a petroleum ether solution of vaseline. (Proper vaseline application was critical to membrane stability.)

Ten to fifteen microliters of the hexane suspension to be tested were deposited over the surface of a shallow layer of solution in each compartment. (The solutions did not touch

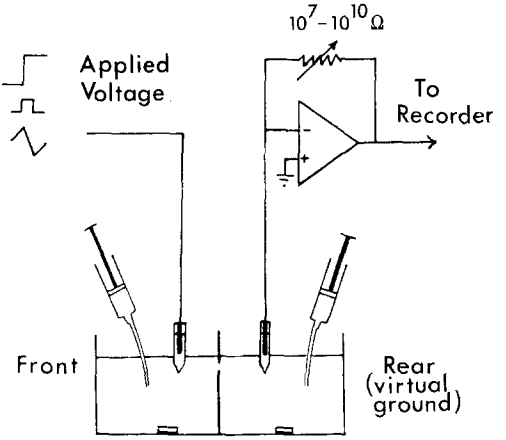

Fig. 1. Schematic diagram of the experimental arrangement for stimulating and recording across planar bilayers. The front and rear compartments of the chamber are separated by a Saran Wrap partition (*heavy line*) containing the planar lipid bilayer membrane (*thin line*). The syringes are used during membrane formation to raise or lower the levels of the solutions in each of the two compartments. A single pair of calomel electrodes, used for both voltage stimulation and current recording, are connected to an operational amplifier (Teledyne Philbrick number 100301) whose output feeds into both a strip chart recorder (Kipp and Zonen BD-8) and an oscilloscope. The voltage source was usually a function generator (Wavetek model 180). Magnetic bars at the bottom of each compartment could be used to stir the solutions

the partition, thus avoiding contact of the hexane with the vaseline coating the partition.) After a few minutes, to allow for evaporation of the hexane, the level of the rear solution was raised slightly above the hole in the partition. The level of the front solution was then slowly raised, and membrane formation was followed by monitoring the increase in capacitance (Montal, 1974*a*). Membrane formation was also monitored optically with a dissecting microscope (20 ×). The interface at the upper edge of the growing membrane forms a characteristic crescent which disappears when the membrane breaks. By following the upward motion of the crescent above the top of the hole, one knows when the membrane is fully formed. All experiments were done at room temperature.

All electrical measurements were made by voltage clamping and recording with a single pair of saturated calomel electrodes contacting the solutions through saturated KCl junctions (*see* Fig. 1). Either voltage steps or 5 mHz triangular voltage waves (1 triangle/200 sec) were used as stimuli; the resulting response was fed from the current output of the voltage-clamp amplifier into both an oscilloscope and a pen recorder. (With the exception of those measurements made during membrane formation, capacitance current was too rapid or too small to be observed under the recording-conditions, and it does not appear in any of the records reproduced in this paper.)

Membranes made in symmetrical salt solutions generally were of low conductance with very few channels. On the other hand, membranes made in salt gradients [e.g., 1 M KCl on one side and 0.1 M KCl on the other (both sides also containing 5 mM $CaCl_2$ to improve membrane stability)] had many more channels. (Successful experiments were also obtained with Na^+ or Li^+ as the cation and Br^-, CH_3COO^-, or $SO_4^=$ as the anion.) Membranes made in 0.1 M $CaCl_2$ on one side and 0.01 M $CaCl_2$ on the other also yielded large numbers of channels. If the solutions were made symmetrical after the membrane was made, a large

number of channels still remained. [Membranes made from only asolectin, with no protein additives from paramecia, generally had very low conductance (≈ 10 pmho) and never contained voltage-dependent anion-selective channels.] The potential of the rear chamber is defined as 0; thus positive current flows from front to rear.

Negative Staining

A drop of sample was placed on a carbon-coated formvar-coated grid, left for about two min, and then "sponged off" by touching the edge of the grid to Whatman #50 filter paper. A drop of 1% PTA (phosphotungstic acid) adjusted to pH 7.0 was then placed on the grid and quickly sponged off. Grids were viewed in a Siemens Elmiskop Electron Microscope.

Miscellaneous

Protein was measured by the method of Lowry, Rosenbrough, Farr and Randall (1951). Asolectin, a crude mixture of soybean phospholipids, was purchased from Sigma Chemical Co. (St. Louis, Mo.) as lecithin Type II-S and treated with acetone and ether as described by Kagawa and Racker (1971). All other chemicals used were reagent grade.

Results

Bilayers formed with the mixed membrane fraction or some of the more purified fractions (*see* Table 1) have a voltage-dependent conductance that is highest near $V=0$ and falls to a minimum at positive and negative voltages greater than 40 mV[2]. When the voltage across the bilayer is suddenly changed from 0 to 40 mV, the current decays over many seconds from an initial high value (which reflects the conductance at 0 mV) to a smaller steady-state value (Fig. 2*a*); i.e., there are conducting elements in the membrane which turn off slowly when the voltage is increased. If the voltage is returned to 0 for only 1 msec, the initial current level upon returning the voltage to 40 mV shows that already about 30% of the voltage-dependent conduction elements have turned on at 0 mV (Fig. 2*b*); if the voltage is left at 0 mV for 500 msec, virtually all of the voltage-dependent conducting elements have turned on (Fig. 2*c*). Thus, conductance turn on (at 0 mV) is much faster than conductance turn off (at 40 mV).

The steady-state current-voltage ($I-V$) characteristic is shown in Fig. 3*a*, and from this the steady-state conductance-voltage ($G-V$)

2 The *total* membrane conductance could be due to many types of conducting elements. Since parallel conductances sum, we can focus on the voltage-dependent conductance and ignore the rest.

Table 1. Localization of VDAC activity

	Fractions from the sucrose gradient								Purified pellicle	Cilia
	Top	30–35	35–40	40–45	45–50	50–55	55–60	Pellet		
Total sedimentable[a] protein (mg)	<0.1	<0.1	<0.1	0.7	1.0	1.6	1.8	0.4	8	4
Electronmicroscope observations										
Mitochondria	−	−	−	+++	+++	++++	+	±	+	
Trichocysts	−	−	−	+	±	−	++	++	+	
Trichocyst tips	−	−	−	−	−	−	−	++	−	
Pellicle fragments	−	−	−	−	−	−	+	±	+++	
Unidentified material	−	++	++	+	+	±	+	+	+	
VDAC Activity[b] (±SD)			3 ±0.5	250 ±150	300 ±300	500[c] ±300	3 ±4	*N.D.*[d]	20[e] ±20	*N.D.*[d]
VDAC Activity % of total			<0.1	12	21	55	<1	<1	11	<1

[a] The protein was measured after centrifugation and washing as in *Materials and Methods.*

[b] VDAC activity is the number of channels observed in planar bilayers made from a 1.4% suspension of the appropriate fraction (treated as described in *Materials and Methods*) in hexane. The bilayers were formed in 1.0 M KCl, 5 mM $CaCl_2$ *vs.* 0.1 M KCl, 5 mM $CaCl_2$.

[c] The membranes formed from a 10-fold dilution (with 1.4% asolectin) of this suspension had 80±30 channels.

[d] *N.D.*-none detected; in the case of the pellet, the baseline conductance could have hidden some VDAC.

[e] Some mitochondria appear to be attached to the pellicle.

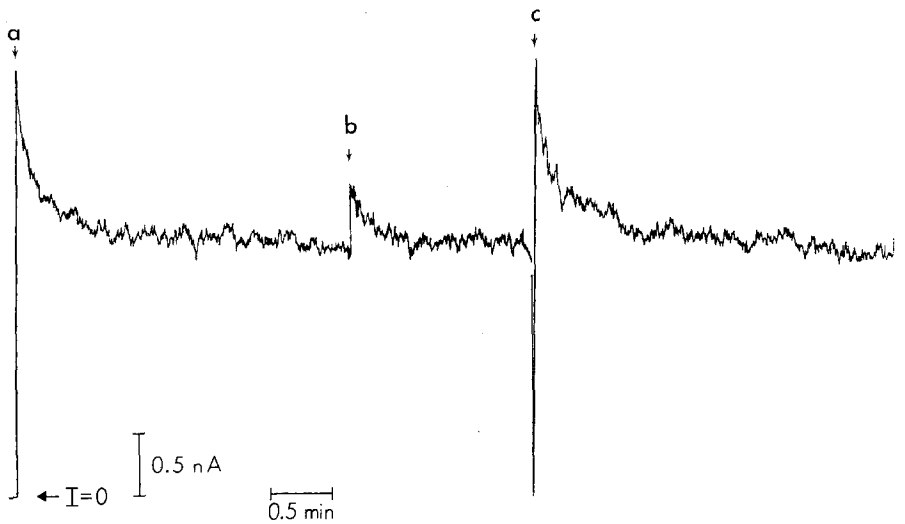

Fig. 2. Current responses of a parameciumstoffs-containing asolectin bilayer to voltage pulses. The membrane was formed in 0.1 M $CaCl_2$, using fraction 45–50 of Table 1. At point *a* the voltage was changed from 0 to 40 mV. The initial high value of current (which reflects the conductance at 0 mV) decays over many seconds to a smaller steady-state value (i.e., to a smaller conductance). At point *b* the voltage is reduced to 0 for 1 msec and then returned to 40 mV. [The current drop to $I=0$ when $V=0$ is not seen because of the slow response time of the recorder (600 msec full scale).] Note that the initial current value when the voltage is returned to 40 mV is larger than the previous steady-state value, thus showing a substantial turn-on of conductance during the 1 msec at $V=0$. At point *c*, the voltage is reduced to 0 for 500 msec and then returned to 40 mV. We see that the initial current value when the voltage is returned to 40 mV is the same as at *a*; thus, complete turn-on of conductance has occurred during the 500 msec at $V=0$

characteristic (Fig. 3*b*) (in symmetric solutions) is determined from the relation:

$$G=\frac{I}{V}. \tag{1}$$

The figure shows a high conductance region around $V=0$ and two lower conductance regions at positive and negative voltages, with each of the latter joined to the high conductance region by a transition or switching zone. The steepness of the transition from the low conductance region to the high conductance region reflects the voltage sensitivity of the conducting element.

The molecular basis for the voltage-dependent conductance is the presence of channels with voltage-dependent lifetimes. The current records in Fig. 4 show discrete jumps characteristic of channels. Furthermore, we see that channel lifetime varies with voltage. At 6 mV, the channels spend nearly all the time in the "open" state; at $V=20$ mV, they spend

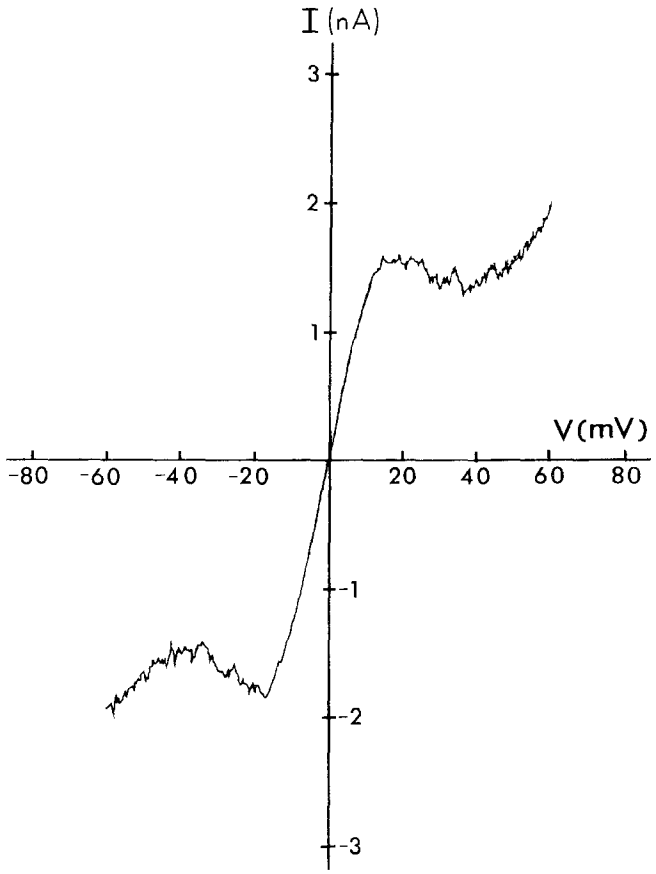
I (nA)
3
2
1
V(mV)
-80
-60
-40
-20
20
40
60
80
-1
-2
-3

Fig. 3a

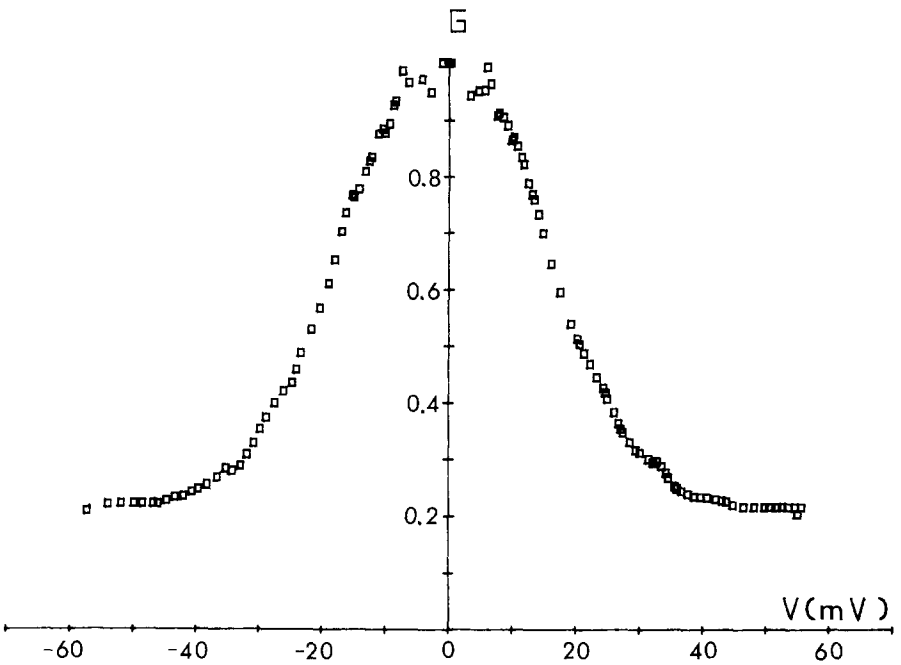
G
0.8
0.6
0.4
0.2
V(mV)
-60
-40
-20
0
20
40
60

Fig. 3b

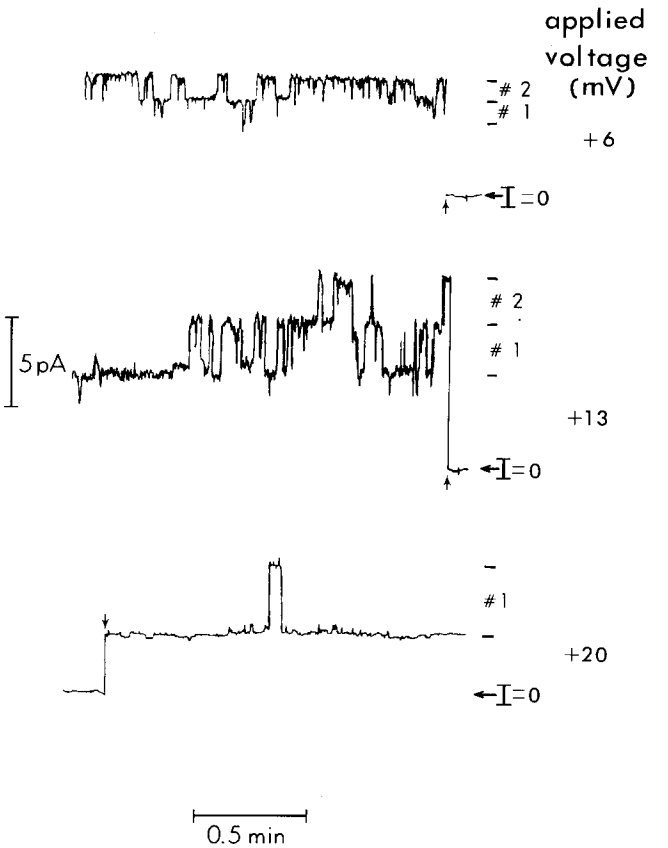

Fig. 4. Current (conductance) fluctuations at several voltages due to the presence of two channels in the membrane. [Time is Hebraic (proceeding from right to left).] The upward arrows mark the time when the voltage was switched from 0 to the designated value; the downward arrow marks the time when the voltage was switched from 20 mV to 0. The two conductance levels are indicated by #1 and #2. The conductance of a single channel is about 200 pmho and is voltage-independent. (The size of the current fluctuations is directly proportional to the applied voltage and hence is smaller at 6 mV than at 20 mV.) Note that at 6 mV both channels are almost always open; at 13 mV usually only one channel is open at any given time; at 20 mV a channel is rarely open. (Occasionally channels are observed whose conductance value is less than that of the usual single channel.) During the course of the experiment the ohmic conductance was changing. The differences in its value seen in these records are irrelevant to our concerns. The bilayer, made from the "mixed membrane fraction" (*see Materials and Methods*), separates 20 mM $MgCl_2$ in the front compartment from 20 mM $CaCl_2$ in the rear

Fig. 3. (*a*) The steady state current-voltage ($I-V$) characteristic of the system. The membrane was made in symmetrical 0.1 M $CaCl_2$, using fraction 40–45 of Table 1. The current response is to a 5 mHz triangular wave of voltage. Only the current responses in the "turn-on" direction (high voltage to 0) were used to generate the $I-V$ characteristic. That this is indeed a *steady-state* characteristic was verified by obtaining the identical plot with a 0.5 mHz triangular wave. [The slow "turn-off" kinetics (*see* Fig. 2) precluded our obtaining steady-state current values from voltage scans of increasing potential.] (*b*) The steady state conductance-voltage ($G-V$) characteristic of the system. The points were calculated from the data in Fig. 3*a* using the relation $G=I/V$. The peak conductance (151 nmho) has been normalized to 1. The voltage-dependent part of this total conductance was 118 nmho and the voltage-independent baseline was 33 nmho

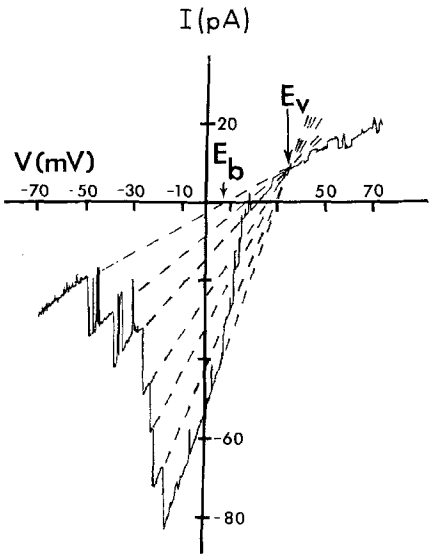

Fig. 5a

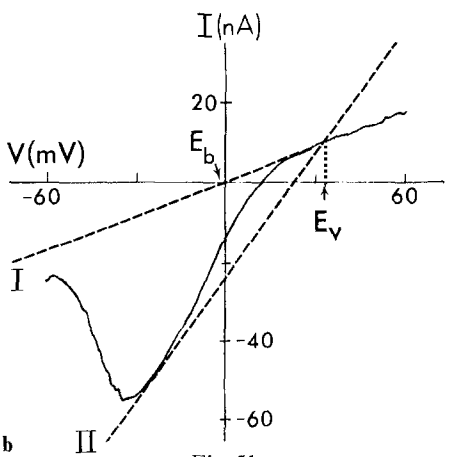

Fig. 5b

Fig. 5. (*a*) A current-voltage record obtained on a membrane containing a few (6) channels in the presence of a salt gradient. The bilayer, made using the "mixed membrane fraction" (*see Materials and Methods*) separates 80 mM $CaCl_2$ in the front compartment from 20 mM $CaCl_2$ in the rear. The record was generated by a 5 mHz triangular wave traveling from +70 to −70 mV. Note that the extrapolations of all the linear *I–V* regions (i.e., those regions where the conductance is not changing) pass through a single point. The voltage corresponding to this point is the reversal potential, E_v, for a channel (*see Appendix A*). E_b is the reversal potential of the voltage-independent baseline conductance, and is close to zero. (*b*) A current-voltage record of a membrane containing many channels in the presence of a salt gradient. The bilayer, made using fraction 50–55 of Table 1, separates 1.0 M KCl (5 mM $CaCl_2$) in the front compartment from 0.1 M KCl (5 mM $CaCl_2$) in the rear. The record was generated by a 5 mHz triangular wave traveling from +60 to −60 mV. Dashed line I

very little time in this state. Qualitatively, this behavior accounts for the macroscopic $G-V$ characteristic of Fig. 3*b*. From Fig. 4, the conductance (or rather, the difference in conductance between the open and closed state) of a single channel is seen to be 200 pmho (with 40 mM Cl^- on both sides of the membrane).

It is convenient to study single (or few) channel membranes in the presence of an ionic gradient with the same voltage scanning technique used to generate Fig. 3*a*. The analysis of the $I-V$ relations is simple and powerful, yielding channel selectivity, single channel conductance, and providing information on the "ohmic" or "nonohmic" nature of the open conductance state. This analysis (which is explained in *Appendix A*) is illustrated in Fig. 5*a*. A family of lines is generated in the $I-V$ plot, each line differing in slope by approximately 300 pmho, the conductance of a single channel in the asymmetric $CaCl_2$ medium. When the lines are extrapolated they cross at one point, where $V=E_v$, the reversal potential of the channel. In the 4-fold gradient of $CaCl_2$, the reversal potential of the single channel is +35 mV, that expected for a channel ideally selective for Cl^- over Ca^{++}. We also see from Fig. 5*a* that the open conductance state is ohmic.

Knowledge of the reversal potential permits the $I-V$ characteristic of a membrane with many channels in the presence of an ionic gradient to be treated in the same manner. (The reversal potential may be obtained from single channel data as above or from the current transients in records such as those in Fig. 2 taken in the presence of a salt gradient.) Fig. 5*b* shows such a result; the $I-V$ characteristic reflects a simple switching between the low background conductance state (line I) and the high-conductance state (line II). The total active conductance in this membrane, computed as the difference between the conductance in the low and high conductance state, indicates the presence of about 1000 channels.

was drawn by extrapolating the linear portion of the experimental curve in the first quadrant. Dashed line II is tangent to the experimental curve at the point of maximal conductance; it intersects line I at a point whose voltage is +35 mV, and this is E_v. [The slope of line II should represent the maximum membrane conductance. If a clear linear region existed at maximal conductance in the experimental curve, no additional information would be required to draw it. Since such a region does not exist, in practice E_v is determined from independent single channel data (such as those in Fig. 5*a*) and line II is drawn tangent to the experimental curve through the point on line I corresponding to E_v.] By subtracting the slope of line I from that of line II, one obtains a value of 750 nmho for the maximum value of the voltage-dependent conductance. Hence, this membrane contains about 1000 channels (single channel conductance $\approx$750 pmho)

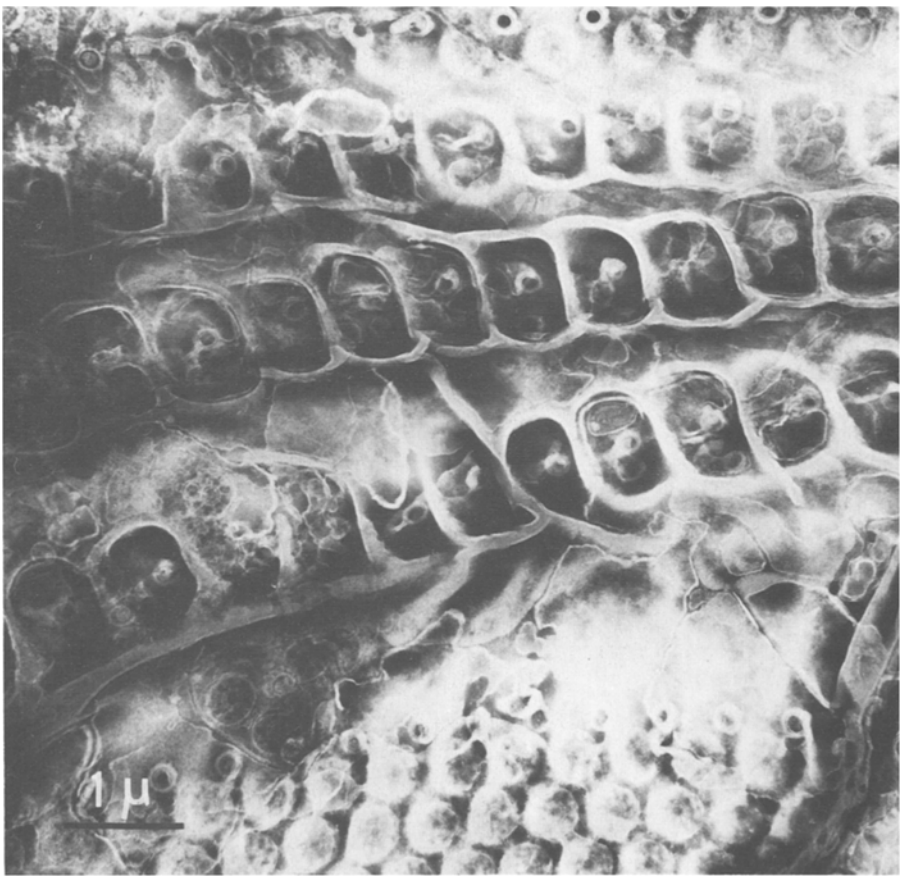

Fig. 6. Electronmicrographs of negatively stained fractions. (*a*) pellicle fraction; (*b*) fraction 50–55 at low power; (*c*) fraction 50–55 at high power; (*d*) the pellet. *See* text for description of these micrographs

Using different single salt gradients, we obtain the selectivity sequence:

$$Cl^- \approx Br^- > CH_3COO^- > K^+ \approx Na^+ > SO_4^{=} \gg Ca^{++} \approx 0.$$

Ca^{++} is essentially impermeant, whereas K^+ is (from the constant field equation) about 7 times less permeant than Cl^-. [The reversal potential is about 35 mV with a 10-fold gradient (1 M *vs.* 0.1 M) of KCl across the membrane.] Hence, we are dealing with a *voltage dependent anion-selective channel* (VDAC).

Origin of VDAC

Since most channel-formers incorporated into planar bilayers have been of microbial origin, and since the paramecia were grown on bacteria,

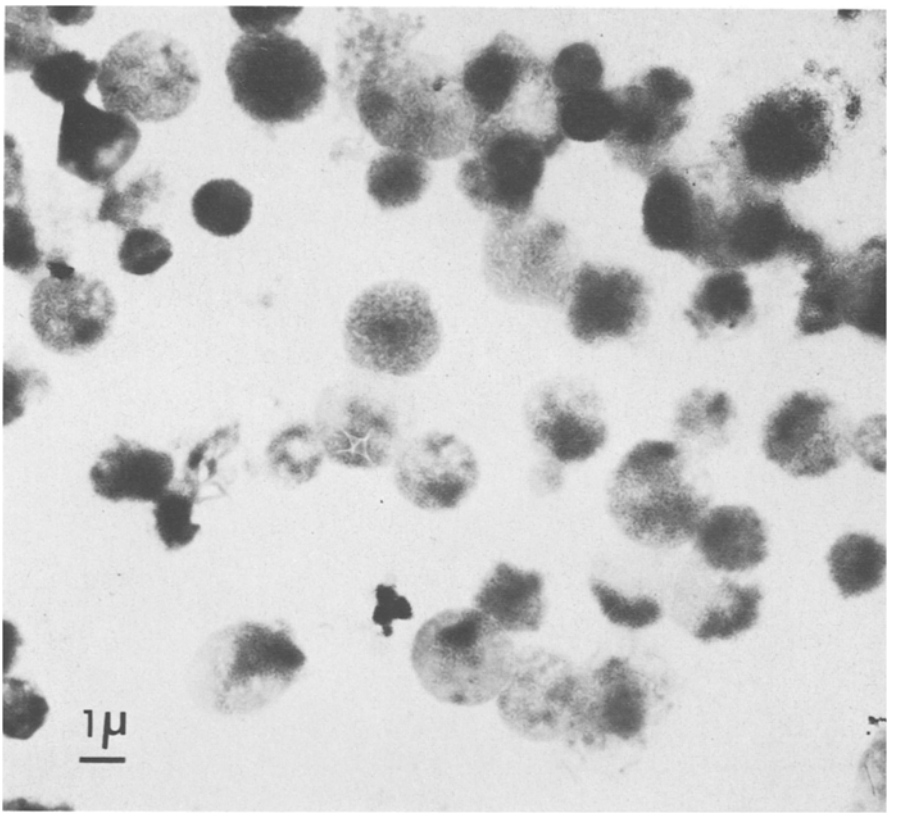

Fig. 6b

we were concerned that a bacterial contaminant was responsible for VDAC. This possibility was excluded by the finding that VDAC was obtained at comparable yield from the mixed membrane fraction of paramecia grown on an autoclaved axenic medium. This finding also excluded the possibility that the axenic medium itself is a source of VDAC, since autoclaved mixed membranes have no activity (i.e., VDAC is heat denatureable). In addition, good VDAC activity was obtained from paramecia grown on a defined medium (containing amino acids, nucleotides, vitamins, fatty acids and "animal cephalin"). In order to localize the source of the voltage-dependent channels, the purified pellicles and the fractions from the pellicle-free supernatant were examined by negative staining for their organelle content and assayed for their channel content. Fig. 6*a* shows the characteristic ribbed appearance of the pellicle, the triple-membraned structure which envelopes the paramecium and includes the outer membrane. The striations on the pellicle

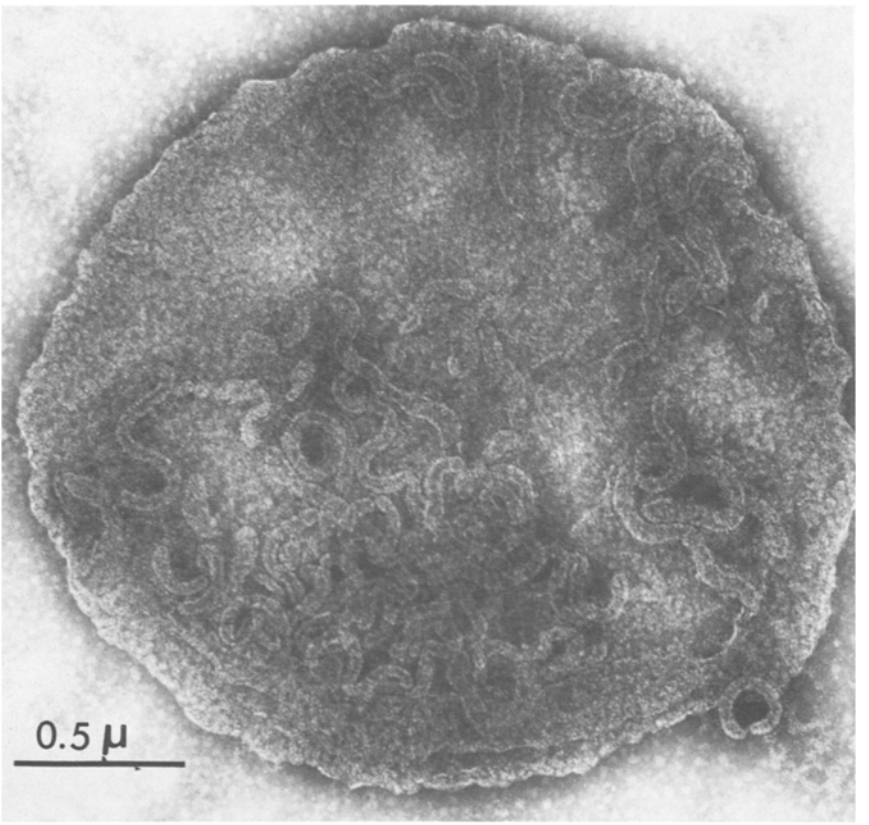

Fig. 6c

are easily visible with the light microscope. Figs. 6*b* and 6*c* are low and high power views, respectively, of fraction 50–55. The vermicular appearance of the organelles is characteristic of paramecium mitochondria; thus, this fraction appears to be nearly pure mitochondria. The pellet from the sucrose gradient, Fig. 6*d*, has relatively few mitochondria and contains large numbers of trichocysts, both sprung (the striated structure) and unsprung, and trichocyst tips.

A large variability (reflected in the large standard deviation in Table 1) exists in the number of channels obtained in membranes formed from a given fraction. Nonetheless, it is evident that those fractions that have a large number of mitochondria also have the greatest number of channels. Fractions containing other organelles but few mitochondria have few channels. About 60% of the VDAC activity is localized in the 50–55% sucrose fraction, which is nearly pure mitochondria.

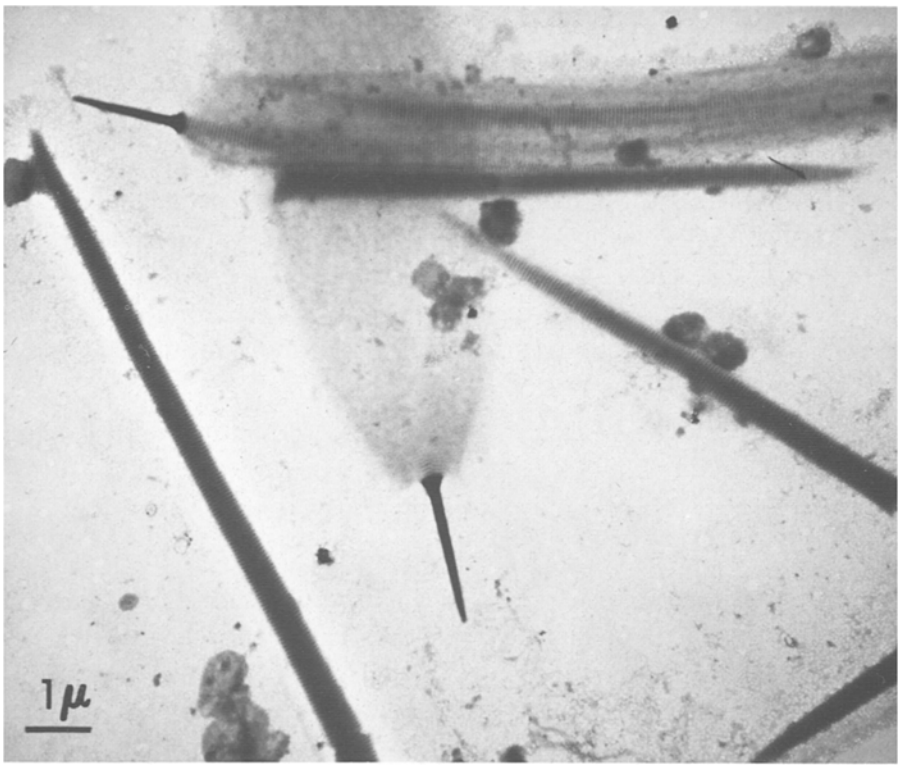

Fig. 6d

Discussion

We have qualitatively described the electrical properties of lipid bilayer membranes containing voltage-dependent, anion-selective channels (VDAC) obtained from paramecium mitochondria. The channels completely discriminate between Cl^- and Ca^{++} and are about 7 times more permeable to Cl^- than to K^+ or Na^+. They have two switching regions, being open around $V=0$ and closing with both positive and negative voltages. The single channel conductance in the open state is about 750 pmho [3]; membranes with voltage-dependent conductances as large as 1 μmho (i.e., membranes containing about 1000 channels) were obtained in some cases.

There was considerable variability in the number of channels obtained even from successive membranes formed from the same monolayer. Occa-

3 This value is obtained in a 10-fold KCl gradient (1 M *vs.* 0.1 M). In a 10-fold $CaCl_2$ gradient (0.1 M *vs.* 0.01 M) the single channel conductance is about 300 pmho, and in symmetrical 0.1 M $CaCl_2$, it is 600 pmho.

sionally during an experiment on a given membrane, the number of channels might suddenly increase or decrease by a large increment, suggesting that the channels are incorporated into the membrane as patches or clusters. Despite this *quantitative* variability, the system is highly reproducible *qualitatively*. We have obtained VDAC in hundreds of membranes using dozens of more or less purified mitochondrial preparations combined with an excess of asolectin. We have never seen VDAC in bilayers made from asolectin alone; indeed, for a given paramecium membrane fraction, the number of channels obtained in a bilayer is roughly proportional to the ratio of protein to asolectin in the membrane-forming solutions.

Analysis of the Voltage Dependence

Because we are not dealing with a chemically pure system, it is difficult to estimate the conductance of the channel in the closed state. The voltage-*independent* conductance (which is slightly cation selective) is probably at least partly due to other conductance elements in the membrane, since its magnitude is not a constant fraction of the voltage-dependent conductance. Regardless of its origin, we can subtract it from the total conductance and focus only on the voltage-*dependent* aspect of the total membrane conductance, which we call G_v. Graphically, this simply means that the $G-V$ characteristic in Fig. 3*b* is moved downward so that G_v assymptotes to zero, instead of to some finite value, for large V's [4].

The $G-V$ characteristic in Fig. 3*b* reveals a rather steep dependence of conductance on voltage. A simple two-state model for the channel gives the expression (*see Appendix B*):

$$\frac{G_v}{G_{\max}-G_v}=e^{-nq(V-V_0)/kT} \tag{2}$$

where $G_{\max}$ is the maximum value of G_v (it occurs around $V=0$), n is the equivalent number of charges that must be moved to close or open a channel, V_0 is the switching voltage (at $V=V_0$ there is an equal number of channels in the open and closed states), q is the electronic charge, k is Boltzmann's constant, and T is temperature in degrees Kelvin. Fig. 7

4 The voltage-independent conductance is not always the same for positive and negative voltages.

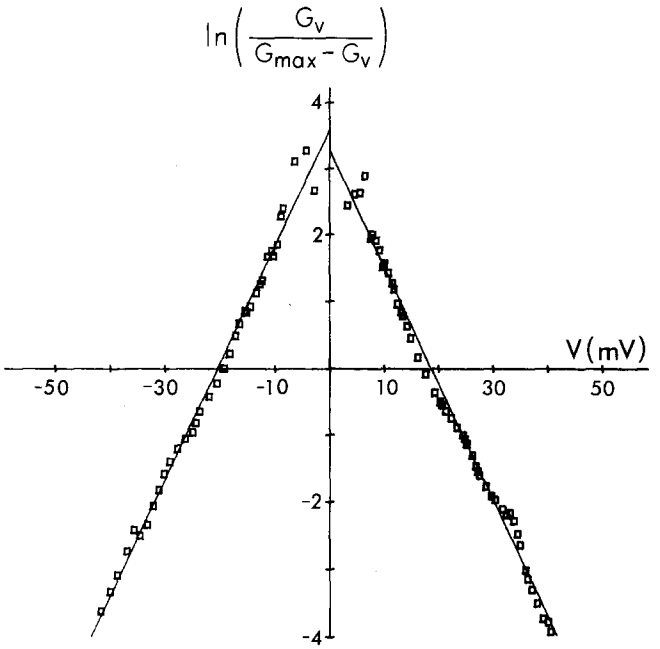

Fig. 7. Plot of $\ln \frac{G_v}{G_{max}-G_v}$ *vs.* V. The points are a replot of those in Fig. 3*b*, where G_v is the conductance at any point in Fig. 3*b* minus the ohmic conductance (the conductance at large values of V). G_{max} is the maximal conductance in Fig. 3*b* (it occurs at $V=0$) minus the ohmic conductance. We note that the points lie on straight lines, as predicted by a two state model for the channel (*Appendix B*). The slopes of these lines are steep (n of Eq. (2) is 4.5); that is, the channels are strongly voltage-dependent

is a plot of $\ln \frac{G_v}{G_{max}-G_v}$ *vs.* V and gives a straight line as predicted by Eq. (2); from the slope and intercept we obtain $n=4.5$ and $V_0=\pm 20$ mV. (Values of n range from 4 to 8; values of V_0 range from ± 15 to 20 mV.) A value of $n=4.5$ means that at voltages where $G_v \ll G_{max}$, G_v changes e-fold for every 5.5 mV shift in membrane potential.

Although plots of $\ln \frac{G_v}{G_{max}-G_v}$ *vs.* V give straight lines, we cannot place much significance on the value for n. In the first place, there may be two populations of channels, those that came from the monolayer in the front and those that came from the monolayer in the rear, oriented in opposite directions. Data from membranes with only one channel show that a single channel turns off for both positive and negative voltages, but the functional dependence of channel life time on voltage is probably not symmetrical about $V=0$. In the second place, the clearly multi-exponential kinetics associated with the turn-off conductance (*see* Fig. 2)

implies that there may be more than one closed state, contrary to the simple model we have considered. Clarification of these and other issues must await more extensive single channel data and purification of the system, both of which are in progress.

Method of Incorporating Channels into Bilayers

The method we have used for transferring channels from natural to artificial membranes may have general utility. Although the exposure of a biological system to hexane might be expected to denature the material, observations with several systems indicate that the procedure we have employed is nondenaturing. Thus, VDAC is active after this treatment, although it is heat-denaturable; the ouabain sensitive $Na^+ - K^+$-ATPase from pork cerebral cortex microsomes retains full activity after suspension in hexane according to our method [it was assayed by blowing off the hexane and resuspending the ATPase in water (Joshua Zimmerberg, *unpublished observations*)]; the acetylcholine receptor from electroplax retains its specific reactivities toward an affinity label and α-neurotoxin after this treatment (Arthur Karlin, *unpublished observations*). Possibly the excess phospholipid coats and protects the hydrophilic portions of these molecules. Whatever the mechanism of preserving activity, this method may prove useful in reconstituting other transport systems in planar bilayers.

Comparison with EIM

There are interesting similarities between these voltage-dependent anion-selective channels, obtained from mitochondria, and excitability inducing material (EIM), obtained from bacteria (Ehrenstein, Lecar & Nossal, 1970). In particular: (1) the $G-V$ characteristic of both has a maximum at $V=0$ and declines for both positive and negative voltages, and this is a property of a single channel; (2) voltage dependence arises because the lifetime of the open and closed state is a function of voltage; (3) single channel conductances are comparable, although VDAC is anion selective whereas EIM is cation selective. (EIM cation selectivity can be converted to anion selectivity with protamine (Mueller & Rudin, 1968).)

Biological Implications

If we were dealing with voltage-dependent sodium selective channels isolated from nervous tissue, there would be no question about the biological connection. Unfortunately, the $G-V$ characteristics of mitochondria have not been so well studied, and it is also possible that the detailed properties of VDAC depend on its particular lipid environment. It is interesting, however, that Nicholls (1974) has shown that the chloride permeability of brown fat mitochondria, which are naturally uncoupled, is high. In addition, the data of Brierly (1970), Brierly and Stoner (1970), and Weiner (1975) can be interpreted to show that if the mitochondrial membrane potential is discharged, nonrespiring rat liver mitochondria develop a high chloride conductance. It is therefore possible that mitochondria have a voltage-dependent anion-conducting system, which is turned off by the normally high negative internal potential but turned on when this potential falls to low values, and that it is this system that we have stumbled upon[5]. We have recently found voltage-dependent, anion-selective channels with approximately the same single channel conductance and voltage dependence as VDAC in *membranes prepared from rat liver mitochondria*[6].

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Appendix A

Fig. 8 shows how the opening and closing of a single channel appears with the voltage scan. Superimposed on the figure are two lines. Line I is the current-voltage relation which characterizes the closed state when the membrane conductance is at the background level (G_b); it consists of the sum of all the voltage-insensitive conductances and their emfs,

5 It is possible to estimate the density of VDAC in mitochondria as 1 per μ^2 of mitochondrial membrane surface area, on the basis of an average number of 500 channels per $10^4\,\mu^2$ of artificial membrane surface area (taking into account the approximately 25-fold dilution of natural membranestoffs with asolectin).

6 Very little of this activity was found in the microsomal fraction from rat liver.

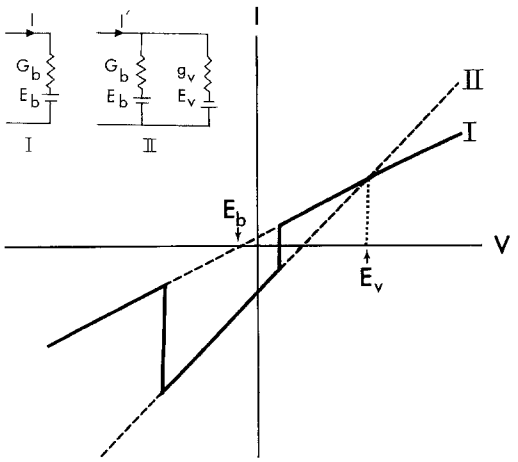

Fig. 8. Idealization of the current-voltage record from a membrane containing only one channel in the presence of a salt gradient. Line I is the $I-V$ characteristic of circuit I, and is appropriate when the channel is closed; line II is the $I-V$ characteristic of circuit II, and is appropriate when the channel is open. The actual $I-V$ relation is shown by the solid line, the jumps occurring when the channel opens or closes. *See Appendix A* for further discussion

and corresponds to circuit I in Fig. 8. Thus,

$$I=\sum_i g_i(V-E_i) \tag{3}$$

which can be written

$$I=G_b(V-E_b) \tag{4}$$

where

$$G_b=\sum_i g_i \quad \text{and} \quad E_b=\frac{\sum_i g_i E_i}{G_b}.$$

Line II characterizes the membrane after a channel has opened and corresponds to circuit II of Fig. 8; then, current flows through both the background conductance and the open channel conductance,

$$I'=G_b(V-E_b)+g_v(V-E_v). \tag{5}$$[7]

The difference in slopes (G_b in the closed state, G_b+g_v in the open state) gives the single channel conductance, g_v. The point at which the two lines cross occurs at $V=E_v$, the reversal potential. With several channels in the membrane, a family of lines is generated, each line differing in

7 Circuit II and Eq. (5) that follows from it are predicated on the assumption that all of the voltage-insensitive conductance (G_b) is in parallel with the voltage-dependent element. If the conductance of "closed" VDAC is a significant fraction of the conductance of "open" VDAC and of different selectivity, the interpretation of E_v is more complex than its simply being the reversal potential of the open channel state.

slope by an integral multiple of g_v and crossing each other at the reversal potential, E_v.

Appendix B

Assume that a channel can exist (for positive voltages) in either an open (o) or a closed (c) state, and that the relative number in each state is given by the Boltzmann distribution:

$$\frac{N_o}{N_c} = \frac{N_o}{N_t - N_o} = e^{-\frac{W(V)}{kT}} \tag{6}$$

where N_o and N_c are the number of channels in the open and closed states, respectively, $N_t (= N_o + N_c)$ is the total number of channels, and $W(V)$ is the voltage-dependent energy difference between the two states. Suppose that it requires the movement of charges associated with the channel to switch the channel from the open to the closed state. If n is the equivalent number of charges which move through the entire transmembrane potential V, then

$$W(V) = n q (V - V_0) \tag{7}$$

and Eq. (6) becomes:

$$\frac{N_o}{N_t - N_o} = e^{-nq(V - V_0)/kT}. \tag{8}$$

Calling g_v the conductance of a channel in the open state, then

$$G_v = N_o g_v \tag{9}$$

$$G_{\max} = N_t g_v \tag{10}$$

and Eq. (8) becomes:

$$\frac{G_v}{G_{\max} - G_v} = e^{-nq(V - V_0)/kT} \tag{11}$$

which is Eq. (2) of the text [8]. A similar analysis can be applied to negative voltages.

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8 The above analysis is the same as that used by Ehrenstein *et al.* (1970) for EIM channels.

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