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PATCH CLAMP ANALYSIS OF A PARTIALLY PURIFIED ION CHANNEL FROM RAT LIVER MITOCHONDRIA

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SUMMARY: A protein fraction isolated from detergent-solubilized mitochondrial membranes by affinity chromatography on immobilized quinine was reconstituted into phospholipid vesicles by detergent dialysis. Vesicles were fused to a diameter of 10 μm or larger by dehydration and rehydration. Patch clamp recordings carried out in detached mode with a symmetrical solution of 150 mM KCl, 5 mM HEPES, and 0.1 mM $CaCl_2$ revealed conductance increments of 140 pS. Transitions of 40 pS were less frequently observed. Control vesicles which lacked protein showed no channel activity. The probability for the 140 pS channel to be open increased with increasing voltage in the range from 20 to 80 mV (positive potentials relative to what was the vesicle interior prior to excision), while the single channel conductance remained essentially constant. The 140 pS channel did not open at negative voltages. The voltage dependence suggests asymmetric incorporation of the 140 pS channel into vesicle membranes during reconstitution.

On the basis of direct and indirect observations of ion transport into and out of mitochondria, the existence of various electrogenic transport catalysts has been postulated. For recent reviews see references 1, 2, and 3. Electrophysiological measurements have indicated the presence of several different ion channels in the inner mitochondrial membrane. These measurements have included patch clamp studies of mitoplasts (4,5,6) and of artificial membranes incorporating mitochondrial membrane fragments or membrane fractions (7,8,9). The channels that have been observed range in conductance from as low as 6 pS to as high as 1.3 nS. A channel exhibiting multiple conductance increments of 220 pS has been postulated to have a role in protein import, because it is blocked by a synthetic peptide having an amino acid sequence identical to that of a mitochondrial targeting signal (8,10). A slightly anion-selective channel that has been observed in mitoplasts and in reconstituted liposomes has been found to have a conductance of 106 pS and to exhibit some voltage dependence (4,9). A 60 kDa protein extracted from mitochondria with ethanol was reported to induce conductance increments of several different magnitudes from 24 pS to 175 pS when added to black lipid membranes (11). These cation-selective

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channels were found to close at voltages above +100 mV or below -60 mV relative to the side of the membrane on which the protein was added (11).

A protein fraction has been isolated from detergent-solubilized mitochondrial membranes by affinity chromatography on immobilized quinine (12). Quinine is an inhibitor of mitochondrial K^+ transport via apparent uniport and antiport mechanisms (13,14,15,16). SDS polyacrylamide gel electrophoresis has indicated that the fraction purified by affinity to quinine contains proteins of about 97, 77, 57, 53 and 31 kDa (17). Phospholipid vesicles reconstituted with the affinity-purified fraction were found to be more permeable to K^+ and to Tl^+, a K^+ analog, than control vesicles which lacked protein (12,17). Analysis (17) of a peptide derived from the most prominent 53 kDa band in the quinine-affinity column eluate indicated an amino acid sequence identical to part of the sequence (18) of aldehyde dehydrogenase, an enzyme unlikely to have a role as a transport catalyst. It has not yet been determined which of the other proteins in the affinity-purified fraction may be involved in the observed cation transport activity. In the present studies, the technique of patch clamping has been used to test for ion channel activity in membranes reconstituted with the affinity-purified protein fraction.

METHODS

Quinine was linked to epoxy-activated sepharose 6B (Pharmacia), columns were loaded with Triton X-100 extracts of rat liver submitochondrial particles and were washed as described (12). The elution medium contained 400 mM KCl, 2 mM Hepes, 1% sodium cholate, 0.5 μg/ml leupeptin and 0.7 μg/ml pepstatin, pH 6.5. The eluate derived from 8 rat livers was concentrated to about 2 ml by ultrafiltration (Amicon YM30 filter). The concentrate (or the same volume of elution medium for controls), was combined in a total of 8 ml with 200 mM octyl glucoside, 15 mg/ml asolectin, and 5 mM Tris P_i, pH 7.5, and dialyzed at 4°C against 2 l of 25 mM KCl with 5 mM Tris P_i, pH 7.5. During 64 hr of dialysis, the medium was changed 4 times, with 8 g BioBeads (BioRad) added in a dialysis sac each time after the initial change of medium at 16 hr. The resulting vesicle suspension was passed through a 2 ml column of BioBeads. SDS PAGE confirmed incorporation of the affinity-purified proteins into the vesicles (17).

Procedures of Criado & Keller (19) were used to fuse vesicles to obtain ones of 10 μm or larger for patch clamping. The vesicle suspension was centrifuged at 50,000 RPM for 1 hr 25 min in a Beckman SW60Ti rotor. A portion of the pellet (1/6 of total preparation) was resuspended with 5 μl of 10 mM MOPS buffer, pH 7.4, plus 10 μl of the same buffer with 10 % ethylene glycol, and deposited as several small droplets on a glass slide. After dehydration for 3 hr at 4°C in a desiccator with desiccant, each dried droplet was overlaid with 20 μl 150 mM KCl, and the slide incubated overnight at 4°C in a closed Petri dish containing a filter paper moistened with 150 mM KCl. Vesicles were rinsed off the slide with 1 ml of solution containing 150 mM KCl, 0.1 mM $CaCl_2$, & 5 mM Hepes(KOH), pH 7.4. Prior to patch clamping, aliquots were transferred to a clean slide, and after 10 min vesicles not adhering to the glass were flushed off. GΩ seals were formed by applying a slight negative pressure to the micropipet. Patches were excised by lifting the pipet away from the vesicle. Only patches with resistances of at least 2 GΩ were used. The reference electrode was a Ag/AgCl wire connected to the bath through an agar bridge containing recording medium. Patch clamp recordings (20,21) were carried out with a symmetrical solution of 150 mM KCl, 5 mM HEPES, & 0.1 mM $CaCl_2$, pH 7.4. A Dagan 3900 patch clamp amplifier in

inside-out mode provided voltage-clamp conditions, as in earlier studies (5). Positive voltages refer to the bath, and correspond to negative pipet potentials. Current was recorded at a bandwidth of 10 kHz via a VR-10A Instrutech digitizer and recorded on VHS tape. Current analysis was limited to a bandwidth of 2 kHz with a Frequency Devices model 902 filter. Data were analyzed on a PC-compatible computer (386) using the PAT Program V6.1 by John Dempster of the University of Strathclyde, U.K.

RESULTS AND DISCUSSION

Fig. 1A shows a portion of a recording made at 30 mV from a membrane patch incorporating the affinity-purified protein fraction. The current transitions are about 4.2 pA. The histogram in Fig. 2A, summarizing the percent time spent at a given current level for the recording partly shown in Fig. 1A, provides a more accurate measure of single channel current than estimates based on individual transitions. For this recording made at 30 mV, the peak of current at about 4.2 pA indicates a single channel conductance of 140 pS. The 140 pS channel was observed in about half of the patches examined from vesicles incorporating the affinity-purified protein fraction.

Fig. 1B shows a recording from a patch of control membrane prepared in the same way as that of Fig. 1A, but omitting protein. The current transitions apparent in Fig. 1A are lacking. The histogram in Fig. 2B confirms the lack of channel activity. No channel activity has been observed in the more than 20 patches of control membranes studied, over the voltage range from -100 to +100 mV.

Fig. 3 summarizes the voltage-dependence of the 140 pS channel in the range from 20 to 80 mV. The probability of the 140 pS channel being open was found to be higher at the upper end of this range of positive potentials relative to the bath (positive on the side of the membrane corresponding to what was the interior of the vesicle prior

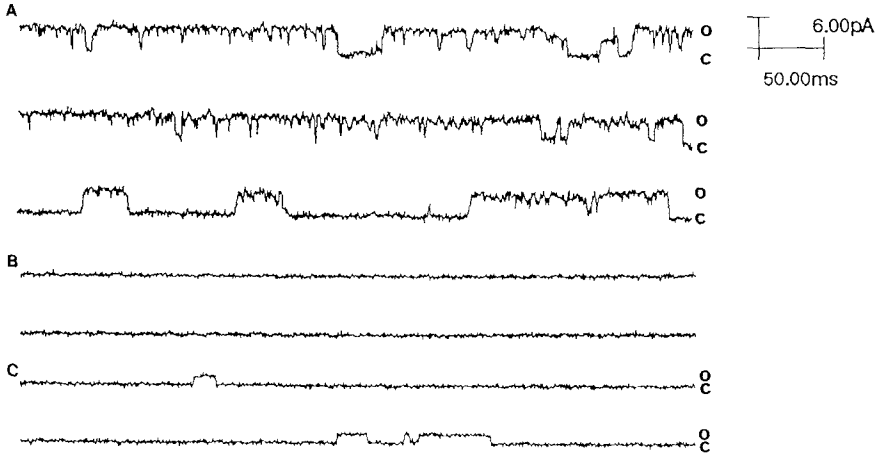

Fig. 1. Continuous patch clamp recordings of current in pA as a function of time in ms. A: Recording at 30 mV (bath potential) from a patch of membrane incorporating the affinity-purified protein fraction, showing the 140 pS channel. B: Recording at 50 mV from a patch of control membrane lacking protein. C: Recording at 50 mV from a patch of membrane incorporating the affinity-purified fraction, showing the 40 pS channel.

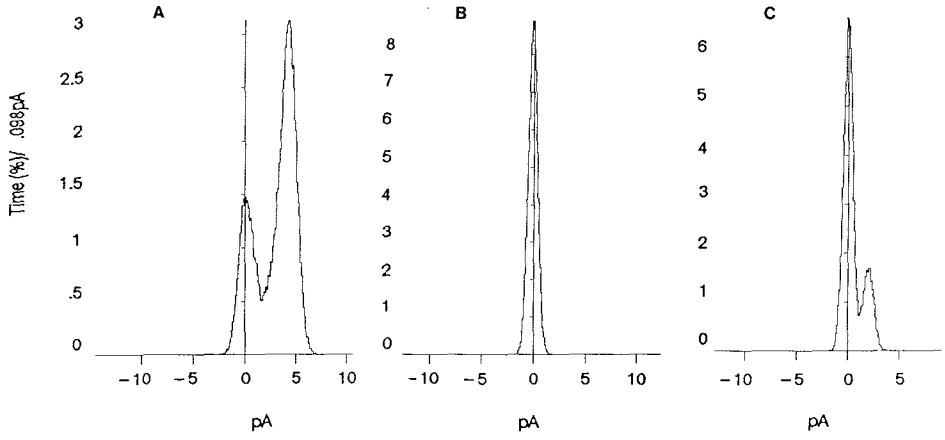

Fig. 2. Summaries of the percent of time at a given pA current for the recordings partially shown in Fig. 1. Recordings were from patches of membrane reconstituted with the affinity-purified protein fraction in A and C or from control membrane lacking protein in B. Voltage (bath potential) was 30 mV in A, and 50 mV in B and C. For each recording summarized, the lowest current seen was arbitrarily set to zero.

to excision, and negative in the interior of the pipet). In addition, in several attempts to study the 140 pS channel at 10 mV relative to the bath no channel openings were seen, and among 38 active patches studied only once was the 140 pS channel observed to open at a negative potential relative to the bath. No systematic variation in single channel conductance was seen over the range from 20 to 80 mV. Calculated values were between 113 and 140 pS, with most values being close to 140 pS. The finding of voltage-dependent gating indicates that the channel must insert into vesicle membranes during detergent dialysis with a non-random orientation, and that the asymmetry is preserved during vesicle fusion. Bacteriorhodopsin also reconstitutes via detergent dialysis with an asymmetric orientation, usually the reverse of the native orientation (22). It has not yet been determined whether the orientation of the reconstituted affinity-purified mitochondrial channel is the same as in the native membrane.

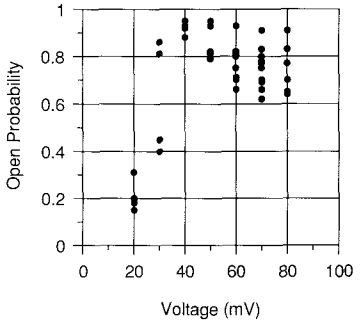

Fig. 3. Voltage-dependence of the 140 pS channel. The open probability is plotted against the voltage in mV (positive relative to the bath). Each data point represents a recording from among 38 patches of vesicles reconstituted with 6 different preparations of the affinity-purified protein fraction.

It is not clear whether the 140 pS channel described here corresponds to one of the channels that have been detected before in mitochondrial inner membranes. The voltage-dependence of the affinity-purified channel is greater, and its conductance higher, than the 106 pS channel observed by Sorgato and coworkers under similar recording conditions (4,9). The lack of variability of conductance increments of the channel described here differs from the behavior of the channel that has been studied by Mironova et al. in black lipid membranes (11). Perhaps the diverse procedures used in these studies may account for some differences in functional properties.

Some patches of membranes reconstituted with the affinity-purified protein fraction exhibit conductance transitions of about 40 pS. A recording from such a patch at 50 mV is partly shown in Fig. 1C and summarized in Fig. 2C. The 40 pS transitions are less consistently seen than those of 140 pS. Many patches exhibit only one or the other type of transition, suggesting that the 40 pS and 140 pS conductance increments are not activities of the same channel. The 40 pS channel exhibits little voltage dependence (data not shown). It has been reported that residual nonionic detergent in liposomes can result in small (e.g. 20 pS) channel-like conductance increments, which are not voltage-dependent (23). Steps were taken to minimize residual detergent in preparing membranes for the present studies (e.g. prolonged dialysis and exposure to BioBeads), but the possibility of contamination with small amounts of detergent cannot be ruled out. It is also possible, since the affinity-purified fraction includes several protein bands, that this fraction may contain more than one ion channel. A quinine-sensitive 45 pS channel, which is not voltage-dependent, has been observed in patch clamp recordings from inner mitochondrial membranes (5,24).

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