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LEAKAGE-CHANNEL CONDUCTANCE OF SINGLE ($Na^+ + K^+$)-ATPase MOLECULES INCORPORATED INTO PLANAR BILAYERS BY FUSION OF LIPOSOMES

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By fusing liposomes which contain in the mean only one pump unit (one intramembranous particle) to planar bilayers, and provoking the ouabain-blockable leakage conductance by the presence of *n*-decane, the predominant unit leakage conductance associated with one pump unit was estimated to be 40–50 pS, indicating the channel nature of the leakage pathway.

The purified Na^+ pump, reconstituted into liposomes, can in the absence of ATP perform as a passive pathway for sodium and potassium ions [1,2]. The slow leakage through this pathway may be accelerated by disabling the ATPase function, for instance by incubation with trypsin [3]. To examine the previous suggestion that the leakage pathway is a channel [3], we have fused Na^+ pump-containing liposomes into planar lipid bilayer membranes and recorded the resulting conductance steps. The putative channel nature of the leakage pathway is of interest, because it opens the possibility that the translocation unit of the Na^+ pump is indeed a gated channel, as previously suggested by several authors (e.g., Ref. 4).

Last et al. [5] have recently shown that the purified pump enzyme, i.e., membrane fragments containing a variable number of pump units [6], can be fused with planar phospholipid bilayers and thereby give rise to large conductance steps which are remarkably variable in size. After fusion, the pump inhibitors ouabain and vanadate cause conductance blockage in large steps, which are comparable in size and variability to those of the fusion events. While the large amplitude of the increasing conductance steps seen on fusion (about 270 pS) may be explained by the simultaneous incorporation of a varying number of conducting pump units, the similar magnitude of the decreasing steps seen after addition of the inhibitors are more difficult to explain. Positive cooperation of clustered, parallel pump units is one of the emerging possibilities. In any case, the conductance steps described by Last et al. [5] are likely to be due to the synchronous performance of several parallel pump units.

To clarify this point we recorded the leakage conductance of single pump units after fusing to planar membranes liposomes which contain in the mean no more than one intramembranous particle per liposome.

Liposomes were prepared from pure phospholipids (80% phosphatidylcholine/20% phosphatidylserine) as described in Ref. 7. Na^+ pump material was prepared from homogenates of the outer medulla of rabbit or porcine kidney and purified by the SDS method of Jørgensen [6], which yields membrane fragments (sheets) containing a number of functional pump units of uniform orientation. The Na^+ pump was recon-

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stituted into single-walled, 90 nm diameter liposomes by detergent solubilisation (1% cholate), lipid addition and dialysis (see, for example, Refs. 7–9). In the liposomes, the pumps are randomly oriented, as judged from the symmetrical distribution of intramembranous particles (IMP) on freeze-fractured concave and convex vesicle halves [9]. By varying the ratio of protein to lipids, liposomes with an adjustable number of intramembranous particles can be prepared [10]. In freeze-fractures, the intramembranous particles have a diameter of 8–10 nm and apparently represent functional pump units [9,10]. By lowering the protein/lipid ratio (prior to dialysis) from 0.3 to 0.05 (w/w), liposomes result which in the mean contain only one intramembranous particle [10]. All active liposome preparations used showed ATP-driven Na^+-K^+ exchange of normal rate, as determined in parallel assays using the method of Ref. 7.

Trypsinization of Na^+ pump protein in membrane fragments was done as described in Ref. 11, using trypsin (bovine pancreas type IV, Sigma) in concentrations of 10 μg/ml and for reaction times of 90 min at 37 °C. By this treatment the catalytic subunit is split, but the fragments apparently stick together, such that the size and density of intramembranous particles observed in the liposomes after reconstitution of trypsin-treated enzyme is unaltered [10]. However, these liposomes are very leaky and the ATPase function is abolished [3].

Phospholipid bilayers were raised in the orifice (2000 μm^2) of a Teflon chamber from a solution of 16 mM phosphatidylethanolamine and 4 mM diphosphatidylglycerol in *n*-decane, using a modified Müller-Rudin technique [12]. As discussed by Last et al. [5] the surprising side-effects of decane are (a) inhibition of the ATPase [13] and (b) inducement of leakage channel properties at the reconstituted pump units [5]. A voltage of $V = +20$ mV (relative to the *trans* side) was usually applied. Fusion of membrane fragments or liposomes into the bilayer was achieved at room temperature as described by Last et al. [5], by adding the material to the *cis* solution together with an excess of NaCl. 50 mM NaCl/50 mM KCl/5 mM $MgCl_2$ was used on the *trans* side, and 130 mM NaCl/50 mM KCl/5 mM $MgCl_2$ on the *cis* side. 25 mM L-histidine buffer (pH 7.15)/1 mM EDTA/1 mM dithioerythritol was present on both sides.

Last et al. [5] estimated conductance as $\Delta I/V$, where ΔI is the amplitude of the current step observed * (Last, T.A., personal communication). It is usual, though, to calculate the chord conductance, i.e., $\Delta I/(V - V_{rev})$, where V_{rev} is the reversal potential. We were unable to measure V_{rev} directly, because the channels closed when V approached zero. However, the current steps did not change appreciably when at +20 mV the Na^+ on the *cis* side was replaced by K^+. Therefore we assumed that the Na^+/K^+ discrimination is negligible and calculated a 'mixed' $V_{rev} = -14.7$ mV, using the sum of Na^+ and K^+ concentrations at each side of the membrane. Conductance was calculated as the ratio $\Delta I/(V - V_{rev})$. In the figures, the current calibration is provided along with values calculated for the conductance steps, because the latter depend on the stipulation that there is no Na^+/K^+ discrimination.

1. When fusing membrane fragments containing purified Na^+ pumps to the black lipid membrane, we observed several increasing conductance steps between 40 and 290 pS. Later on, decreasing steps in this range of amplitudes also occurred spontaneously. Furthermore, addition of vanadate (14 μM) to the *cis* side of the black lipid membrane induced decreasing conductance steps of a similar amplitude. Addition of ATP was without effect. These results confirm those of Last et al. [5].

2. When liposomes containing in the mean about five intramembranous particles were fused to the black lipid membrane, the conductance increased in steps which again ranged between 40 and 290 pS. After fusion, spontaneous and additive off/on conductance steps in the same range of amplitudes were observed (Fig. 1A). Addition of ATP was without effect on the conductance. Also, trypsin treatment of the enzyme prior to reconstitution into the liposomes did not affect the conductance (Fig. 1B).

3. When liposomes containing in the mean about one intramembranous particle were fused to the

* Thus, these authors assumed the reversal potential V_{rev} to be negligible. Had their channels not discriminated between Na^+ and K^+, V_{rev} would have been in the order of −15 mV. In this case, the conductance steps were over-estimated.

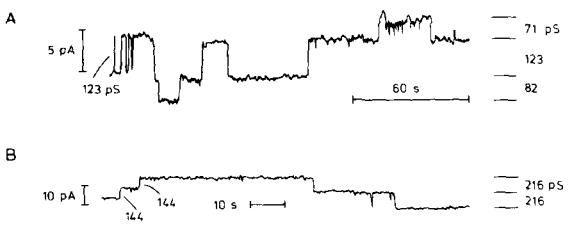

Fig. 1. Additive conductance steps of large and variable size, observed after fusing liposomes (containing in the mean five intramembranous particles) to a black lipid membrane containing *n*-decane. More than one liposome may have fused. For panel B the $(Na^+ + K^+)$-ATPase was treated with trypsin prior to incorporation into the liposomes. A paper chart-recorder with an upper frequency limit of 5 Hz was used. Increasing conductance is plotted upwards.

black lipid membrane, the conductance increased by a single step, which was usually followed by a series of off/on switching events of equal amplitude (Fig. 2). These conductance steps were typically not additive, probably because only one liposome had fused and only a single intramembranous particle had been reconstituted into the black lipid membrane. Subsequent to any-one single fusion event, the steps were remarkably uniform in size, but when comparing different experiments, their amplitudes ranged between 40 and 50 pS. Occasionally conductance steps smaller than 40 pS were observed in preparations which also showed steps in the 40–50 pS range (Fig. 3). The small steps were additive (e.g. Fig. 3, line 4), but not additive to the large steps. Their transitions were often slanted rather than steep (Fig. 3, lines 3, 5 and 8). While it is conceivable that the occasional small steps indicate sublevels of the 40–50 pS conductance, our present material does not permit us to decide this point unequivocally.

Our experiments show that in the black lipid membranes used, a single pump unit is associated with a predominant leakage conductance of 40 to 50 pS at room temperature. While smaller than the 270 pS conductance previously described (see Fig. 6 in Ref. 5), this is still a large value, about 3-times larger than the conductance of the Na^+ channel of the mammalian neuron [14], and about 2-times larger than the conductance of the endplate channel (see, for example Ref. 15).

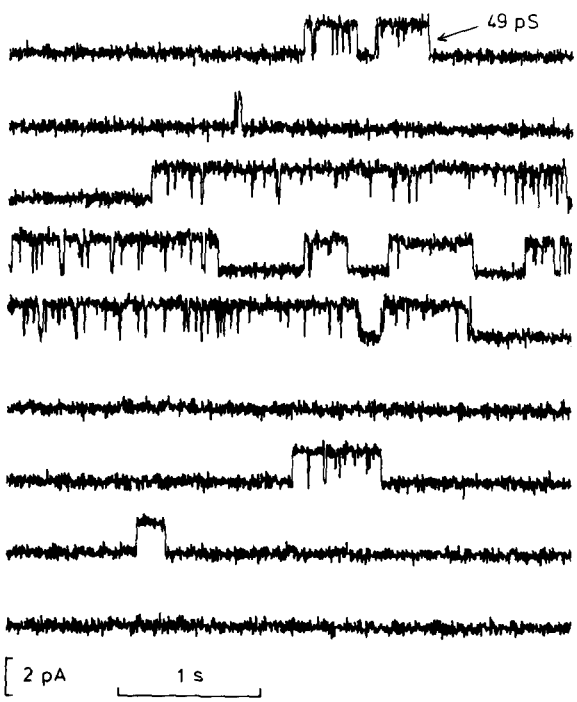

Fig. 2. Non-additive conductance steps of uniform size, observed after fusing a liposome containing probably just one pump unit to the black lipid membrane. The upper levels indicate the open state. Continuous record of 36 s. Upper frequency limit, 210 Hz.

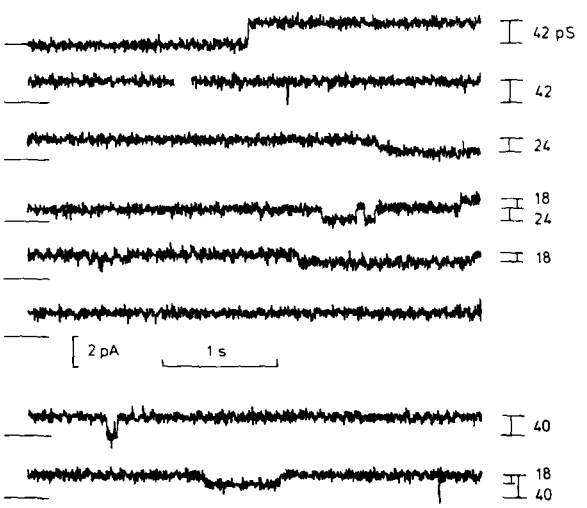

Fig. 3. Records of 24 (above) and 8 s, showing what appear to be sub-levels of the predominant conductance steps, which were 42 and 40 pS in these cases (see right margin). For each trace the apparent closed-state is indicated by a horizontal line on the left. Upper frequency limit, 210 Hz.

Summarizing our results together with those of Last et al. [5]. it appears that the leakage phenomenon comprises: a loss of the absolute requirement for the simultaneous presence of sodium and potassium ions for ion translocation, an increase of the conductance to 40–50 pS per pump, random on/off switching which requires a salt gradient but not the presence of ATP, and a seemingly cooperative switching behaviour if two or more pump units are present in the black lipid membrane and probably in close proximity. Furthermore, the leakage conductance can be blocked by vanadate, by ouabain if a salt gradient is present, and by application of a negative voltage [5].

Clearly the 40–50 pS value is not the conductance of the functional ion pump, but the conductance of a disabled pump, which does not transport actively and does not split ATP. In view of the fact that the leakage conductance is blocked by ouabain and vanadate, the possibility emerges that the functional pump contains one or two channels in its translocation unit, and that these channels are gated or somehow controlled by a regulatory segment of the protein, which binds and breaks down ATP. Interference with this regulation might unmask the channel.

Last et al. [5] suggest that uncoupling of the two units can already come about by the presence of *n*-decane in the black lipid membrane, which is known to increase membrane thickness and thereby to inhibit the ATPase [13]. Inhibition of the ATPase can also be produced by exposure to trypsin [3]. We found that after trypsinization the typical conductance steps can still be demonstrated in the presence of decane. However, to date it remains uncertain whether an increased membrane thickness (decane) causes the leakage phenomenon through uncoupling (possibly through the loss of coordinate, ATP dependent gating), or more directly.

As an alternative to the uncoupling hypothesis, the leak channel might be entirely unrelated to the translocation pathway of the functional pump. For instance, the alteration in membrane thickness might open a gap between membrane-spanning polypeptide strands of the α-subunit. The random open/close switching and the blockability by ouabain and vanadate might be due to ligand-dependent conformational changes of the protein, which affect the conductance of this gap. Therefore, the relation between leakage conductance and functional pump remains to be the central, as yet unsolved problem of the leakage conductance phenomenon. It awaits further experimentation with uncoupled pumps reconstituted into solvent-free black lipid membranes of normal thickness and normal lipid composition.

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