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The ATP-dependent generation of membrane potential by sub-bacterial vesicles from the marine bacterium, *Vibrio alginolyticus*

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Addition of ATP leads to the accumulation of the permeant anion PCB^- by sub-bacterial vesicles from *Vibrio alginolyticus*. This accumulation is caused by $\Delta\psi$ generation by ATPase, the effect being inhibited by CCCP, gramicidin D and DCCD. $\Delta\psi$ values may be increased by incubation of sub-bacterial vesicles at room temperature and with the protein fraction isolated according to Beechey et al. [(1975) Biochem. J. 148, 533–537] from another portion of the sub-bacterial vesicles. $\Delta\psi$ generation is observable only in the presence of Mg^{2+} at high concentrations (optimum $\approx$30 mM). Proceeding from experimental data we assume that Mg^{2+} reduces passive H^+ conductivity of the vesicle membranes. Thus, a $\Delta\psi$-generating ATPase has been shown for the first time in *V. alginolyticus* membranes.

Membrane potential; ATPase; (*Vibrio alginolyticus*)

1. INTRODUCTION

Bacterial cytoplasmic membranes sustain $\Delta\bar{\mu}Na^+$ [1–3]. As a rule, $\Delta\bar{\mu}Na^+$ generation is due to the transformation of the primary $\Delta\bar{\mu}H^+$ by an Na^+/H^+ antiport system. Recently it was shown that the *Vibrio alginolyticus* respiratory chain forms $\Delta\bar{\mu}Na^+$ without participation of the protonmotive force [4,5]. *V. alginolyticus* cells use the $\Delta\bar{\mu}Na^+$ for motility [6,7], solute transport [8,9] and ATP synthesis at alkaline pH [10–12]. On the basis of these data, the Na^+ cycle was postulated as an alternative or an addition to the H^+ cycle [12,13].

At the same time, indications were obtained that *V. alginolyticus* possesses not only $\Delta\bar{\mu}Na^+$ but also $\Delta\bar{\mu}H^+$ generators operative at neutral pH. It was shown that at neutral pH, energy-linked processes are sensitive to the protonophore CCCP and an O_2 pulse leads to a CCCP-sensitive H^+ efflux, the process being accelerated by the permeant cation tetraphenylphosphonium [5]. The primary Na^+ pump was demonstrated for membrane preparations [6,7,14,15] and intact cells [4,5]. $\Delta\bar{\mu}H^+$ generation was, however, observed in intact cells only [5,8]. ATP-dependent $\Delta\psi$ generation by isolated membranes has not yet been demonstrated.

Here, we have obtained the first evidence for $\Delta\psi$ formation during ATP hydrolysis by *V. alginolyticus* sub-bacterial vesicles.

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; PCB^-, phenyldicarbaundecaborane anion; $\Delta\bar{\mu}$, $\Delta\bar{\mu}Na^+$, electrochemical gradients of H^+ and Na^+, respectively; $\Delta\psi$, transmembrane electric potential difference

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2. MATERIALS AND METHODS

The bacterial strain used was *V. alginolyticus* 138-2, which was kindly supplied by Dr H. Tokuda (Chiba University, Chiba, Japan). Bacteria were grown in a complex medium [15] with peptone and were harvested at the late-logarithmic phase. The sub-bacterial vesicles were isolated as described in [6], with some modification. Here 30 mM $MgSO_4$ was included in the media starting from the spheroplast stage. The protein fraction was obtained after treatment of *V. alginolyticus* sub-bacterial vesicles with chloroform essentially as in [16]. The PCB^- concentration was monitored with a phospholipid-impregnated Synpore filter (CSSR) [17]. The Na^+ content was analyzed by flame photometry.

3. RESULTS

Previously, we demonstrated that the procedure for preparation of sub-bacterial vesicles generated NADH-, but not ATP-driven $\Delta\psi$ [6,7]. The continued presence of 30 mM $MgSO_4$ in the medium starting from the stage of spheroplasts allows one to obtain vesicles capable of $\Delta\psi$ generation coupled to ATP hydrolysis. Energy-dependent PCB^- accumulation by the vesicles is shown in fig.1. Gramicidin D and the protonophore CCCP, but not the electroneutral Na^+/H^+ antiporter monensin, prevent PCB^- uptake. Preincubation of sub-bacterial vesicles with the well-known ATPase inhibitor, DCCD, also prevents PCB^- accumulation. Thus, PCB^- uptake after ATP addition reflects $\Delta\psi$ (plus inside vesicles) generation by ATPase.

The amplitude of the ATP-driven $\Delta\psi$ varied in different experiments. The originally low response of some preparations can be increased in two ways (fig.2): (i) by incubation of the vesicles at room temperature (at 25°C maximal activation is achieved over 60 min); (ii) by vesicle preincubation with a protein fraction isolated according to Beechey et al. [16] from another portion of the sub-bacterial vesicles. As can be seen from fig.2, a dramatic increase in PCB^- uptake is observed in both cases.

A decrease in Na^+ concentration from 20 mM to 30 μM (the concentration in the medium without Na^+ additions) does not affect the amplitude of

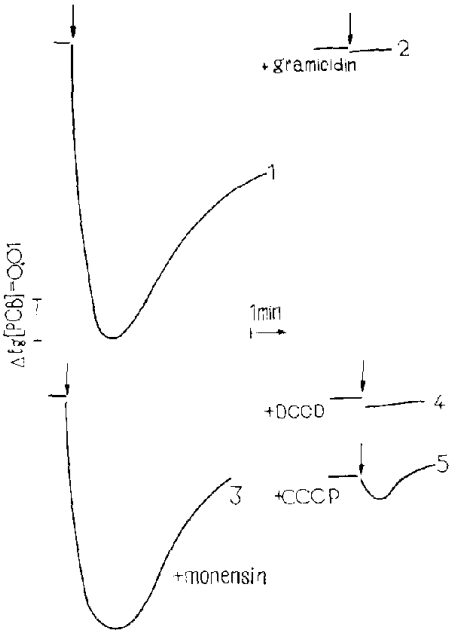

Fig.1. ATP-dependent PCB^- accumulation by sub-bacterial vesicles. The incubation medium contained 50 mM Hepes-NaOH (pH 7.5), 0.1 M sucrose, 30 mM $MgSO_4$, 1 μM PCB^-, 0.25–0.30 mg protein of sub-bacterial vesicles per ml. The reaction was started by the addition of 1 mM Mg-ATP (at the arrow). Additions: (1) none, (2) 2 μM gramicidin D, (3) 2 μM monensin, (4) 80 μM DCCD, (5) 1 μM CCCP.

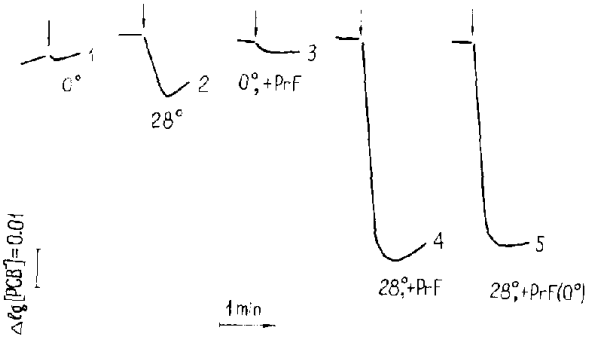

Fig.2. Increase in magnitude of $\Delta\psi$ after incubation at room temperature and with the protein fraction obtained according to Beechey et al. [16]. Sub-bacterial vesicles (37 μl, 25 mg protein per ml) were preincubated for 1 h with 17 μl incubation medium at 0°C (1) or 28°C (2), with 17 μl protein fraction (PrF) at 0°C (3) or 28°C (4). (5) As (4), but protein fraction was preincubated for 1.5 h at 0°C. The reaction was started by addition of 2 mM Mg-ATP (arrow). Incubation mixture, as indicated in the legend to fig.1.

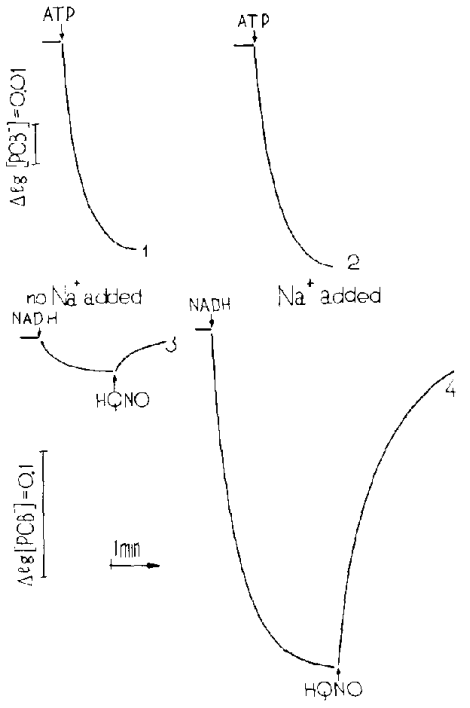

Fig.3. $\Delta\psi$ dependence on Na^+ concentration. The incubation medium contained 20 mM Tris-H_2SO_4 (pH 7.5), 0.1 M sucrose, 30 mM $MgSO_4$, 10 mM $(NH_4)_2SO_4$, 1 μM PCB^- with (2,4) or without (1,3) 10 mM Na_2SO_4. The sub-bacterial vesicles were washed with 10 mM Hepes-Tris (pH 7.5), 0.1 M sucrose, 30 mM $MgSO_4$, and activated, for 1 h at 25°C. Additions: 1 mM ATP (ammonium salt), 1 mM NADH (ammonium salt), 2.5 μM HQNO.

ATP-dependent PCB^- uptake (fig.3). At the same time, under these conditions NADH-dependent PCB^- accumulation is drastically inhibited according to our previous data [6,7].

The present experiments were performed at pH 7.5, however it is well known that Na^+-dependent energetics are much more expressive at alkaline pH [5,9]. Therefore, all experiments were repeated at pH 8.5 or with sub-bacterial vesicles obtained from cells grown at pH 8.5. Practically the same results were obtained in both cases (not shown).

As mentioned above, Mg^{2+} at high concentrations is necessary for ATP-dependent $\Delta\psi$ measurements. The optimal concentration of $MgSO_4$ is 30 mM; Na_2SO_4 at equal ionic strength cannot be substituted for $MgSO_4$ (not shown). To clarify the role of Mg^{2+}, we compared the $\Delta\psi$ supported by NADH oxidation in media with and without $MgSO_4$. As can be seen from fig.4, the inclusion of $MgSO_4$ in the medium dramatically increases the amplitude of the NADH-dependent response and the degree of PCB^- uptake inhibition by CCCP. The most simple explanation of this fact would be an Mg^{2+}-exerted decrease in ion conductivity of the membrane. To confirm this assumption we investigated the influence of DCCD, which reduces the proton conductivity [6,7], on the amplitude of the NADH-dependent response. Despite the very different initial amplitudes, in the presence of DCCD practically the same response was observed in media with and without $MgSO_4$ (fig.4). Thus, Mg^{2+} does not increase the $\Delta\psi$ value of DCCD-treated membranes. It seems that Mg^{2+} and DCCD exert the same action on the membrane conductivity. Thus, the effect of Mg^{2+} at high concentrations on $\Delta\psi$ generation may be accounted for by the reduction of passive proton leakage.

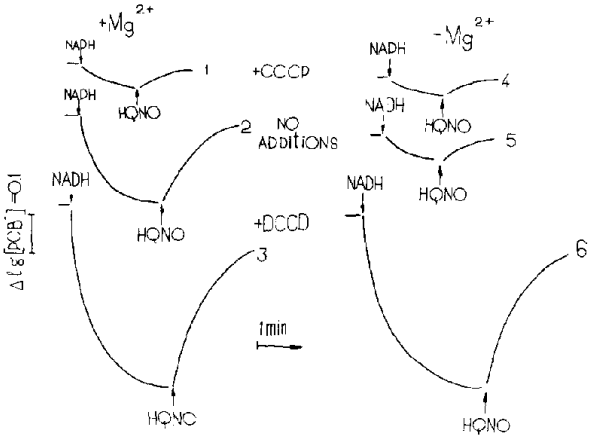

Fig.4. Mg^{2+} effect on $\Delta\psi$ supported by NADH oxidation. Incubation medium: 50 mM Hepes-NaOH (pH 7.5), 0.1 M sucrose, 1 μM PCB^-, with (1–3) or without (4–6) 30 mM $MgSO_4$. Sub-bacterial vesicles preincubated for 1 h at 25°C and then indicated 15 min with 40 μM DCCD. Additions: 1 mM NADH, 2.5 μM HQNO, 5 μM CCCP.

4. DISCUSSION

The addition of ATP to sub-bacterial vesicles causes a decrease in PCB^- concentration in the external medium. Gramicidin D, CCCP and DCCD prevent PCB^- uptake by vesicles. Thus, we have demonstrated for the first time the existence of an

ATPase, which can generate $\Delta\psi$, in isolated *V. alginolyticus* membranes.

ATP-dependent $\Delta\psi$ generation may be measured only in the presence of Mg^{2+} at high concentrations. The experiments shown in fig.4 enable us to assume that Mg^{2+} reduces the proton conductivity of the membrane. It should be noted that the Mg^{2+} concentration optimal for measurements approaches quite closely that in the cells of *V. alginolyticus* [18].

The reason for the influence of Mg^{2+} on proton conductivity is not clear thus far. Probably, Mg^{2+} at high concentrations provides a tight connection between the ATPase and membrane proton channel. Three lines of evidence support this speculation. (i) Sub-bacterial vesicles washed with Mg^{2+}-free medium [6,7] irreversibly lose the ability to generate ATP-dependent $\Delta\psi$. (ii) Even if the washing and incubation media contain 30 mM $MgSO_4$, part of the membrane-bound ATPase is probably lost and can be restored by incubation with the ATPase-enriched protein fraction (fig.2). (iii) DCCD at the same concentration inhibits ATP-dependent $\Delta\psi$ generation and exerts a coupling effect on the $\Delta\psi$ formed during NADH oxidation. Therefore, a partial dissociation of the membrane-ATPase complex may occur; Mg^{2+} may prevent this process.

According to [10,11], $\Delta\bar{\mu}Na^+$ is utilizable for the CCCP-resistant ATP synthesis by *V. alginolyticus* cells. In this respect the nature of the ATPase transferring cation is of particular interest. It was shown that 30 μM–20 mM Na^+ does not exert a pronounced effect on the amplitude of $\Delta\psi$. *V. alginolyticus* cells maintain the intracellular Na^+ concentration at a level of tens of millimoles per litre [8,18]. It is hard to envisage that the K_m of the Na^+-transferring enzyme is as small as 1% of the Na^+ intracellular concentration. Thus, ATP-dependent $\Delta\psi$ formation was hardly due to activity of an Na^+-ATPase. However, our data do not exclude the possibility of two ATPase types (e.g. H^+-ATPase and Na^+-ATPase) in *V. alginolyticus* cells and selective Na^+-ATPase damage during preparation of sub-bacterial vesicles. The latter seems to be rather probable since *V. alginolyticus* is known to produce several proteinases [19].

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