MEMBRANE POTENTIAL GENERATION BY SUBMITOCHONDRIAL PARTICLES ASSOCIATED WITH A LIPID-IMPREGNATED FILTER

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1. Introduction

Transmembrane electrochemical H^+ potential difference ($\Delta\bar{\mu}_{H^+}$) was postulated by Mitchell to be an obligatory intermediate in oxidative and photosynthetic phosphorylation [1]. Progress in the membrane bioenergetics not only verified this postulate but also resulted in elucidation of the fact that $\Delta\bar{\mu}_{H^+}$, like ATP, may serve as a convertible form of energy in the living cell [2]. $\Delta\bar{\mu}_{H^+}$ comprises electric ($\Delta\psi$) and chemical (ΔpH) constituents [1]. Since the electric capacitance of a biomembrane is usually much lower than the pH buffering capacity of the membrane-separated aqueous phases [3,4], it is $\Delta\psi$ rather than ΔpH which should be the initial form of $\Delta\bar{\mu}_{H^+}$ produced by a $\Delta\bar{\mu}_{H^+}$ generator. Therefore, direct $\Delta\psi$ measurements are advantageous in studies on membrane-linked energy transduction.

Since the method of artificial penetrating ions was introduced some ten years ago [5,6], which enabled us to verify the existence of $\Delta\psi$ in a variety of energy-transducing membranes [6–9], we have developed a number of novel facilities in collaboration with Professor E. A. Liberman's group [10–16]. Of these, the association of membranous vesicles with lipid-impregnated filters proved a convenient and potent method of $\Delta\psi$ monitoring, including the measurement of rapid kinetics. This method was used in our studies of bacteriorhodopsin [12,13,15], and the results were confirmed by [19,20]. Chromatophores of *Rhodospirillum rubrum* photosynthetic bacteria were found to be another type of membranous particles to which the method proved applicable [17,18,21]. We reported [22] that submitochondrial particles (SMP) from beef heart could be associated with lipid-impregnated teflon filters and the electrogenic activity of the oxidative phosphorylation machinery was studied in this system. The electric responses of ATPase, cytochrome oxidase, and transhydrogenase were described. The ATPase electrogenic function in the filter-associated SMP has also been investigated using the same method in [23,24].

In [22] porous teflon films from a local source were used. We now report our results with commercially available Mitex filters. With Mitex it is possible to greatly simplify the original procedure [21,22] of particle incorporation into lipid-impregnated filters; also the electrical responses of SMP are found to be more rapid than those in [22,23].

Abbreviations: SMP, submitochondrial particles; DAD, diaminodurene; $\Delta\bar{\mu}_{H^+}$, transmembrane difference in H^+ electrochemical potential; $\Delta\psi$, transmembrane difference in electric potential; PMS, phenazine methosulfate; DCCD, dicyclohexylcarbodiimide

2. Materials and methods

Tap water was deionized, then distilled twice in an allglass apparatus. Tris (Trizma base grade) and soybean phospholipids (phosphatidyl choline, type II-S) were from Sigma. Sucrose (Reachim, 'chemically pure' grade) was recrystallized from ethanol twice-distilled. Other reagents were commercial products of the highest purity available. The Mitex teflon filters with 5 μm pore size were from Millipore.

Phosphorylating, sonic SMP were prepared from beef heart mitochondria essentially after [25].

The electrogenic activity of the filter-associated SMP was measured in a teflon chamber [17,18] that consisted of two 4 ml compartments connecting via

a 4 mm aperture in the insulating wall. The aperture was closed with a teflon filter soaked in a solution of soybean phospholipids in *n*-decane (100 mg/ml), and both compartments were filled with a solution containing 0.25 M sucrose, 50 mM Tris–HCl (pH 7.7) and also 20–30 mM $MgSO_4$ unless indicated otherwise.

SMP (~1.5 mg protein/ml) were added to one of the 2 compartments and particle association with the filter occured upon incubation of the mixture at room temperature; the suspension was stirred continuously with a bar magnet. The procedure was terminated by pouring out the cell contents, to remove the unbound particles, and refilling the cell with a fresh solution.

The electric potential difference across the filter was monitored with a pair of Ag/AgCl electrodes fed into an RFT VA-J-51 Vibron electrometer (GDR) with an actual internal resistance of 10^{11} Ω, connected to a strip-chart recorder. For more details and the layout of the entire measuring system see [17,18].

The electrical resistance of the lipid-impregnated filters was checked routinely before and after recordings of the SMP-induced electrical responses.

All the additions were delivered to both compartments of the chamber unless noted otherwise. The final concentration of ethanol introduced into the reaction mixture with added reagents was ≤1% (v/v).

3. Results and discussion

A typical pattern of the electrogenic activity of SMP associated with a lipid-impregnated teflon filter is given in fig.1. The addition of ascorbate + DAD gives rise to an electric potential difference across the filter (trace 1), negative in the SMP-containing compartment, which is abolished (and prevented, trace 3) by cyanide. ATP added subsequently results in an electric response of the same sign (trace 2), and the addition of DCCD inhibits the electrogenic activity, as also did oligomycin (not shown, [22]). A small increase in the magnitude of the ATP-linked response observed initially after DCCD addition was fairly reproducible and may originate in the 'coupling effect' of the inhibitor. That the electrogenic activity of the SMP linked to the ATPase but not to the cytochrome oxidase reaction is prevented specifically by DCCD can be seen from trace 4 in fig.1.

The rise time of the responses observed with the

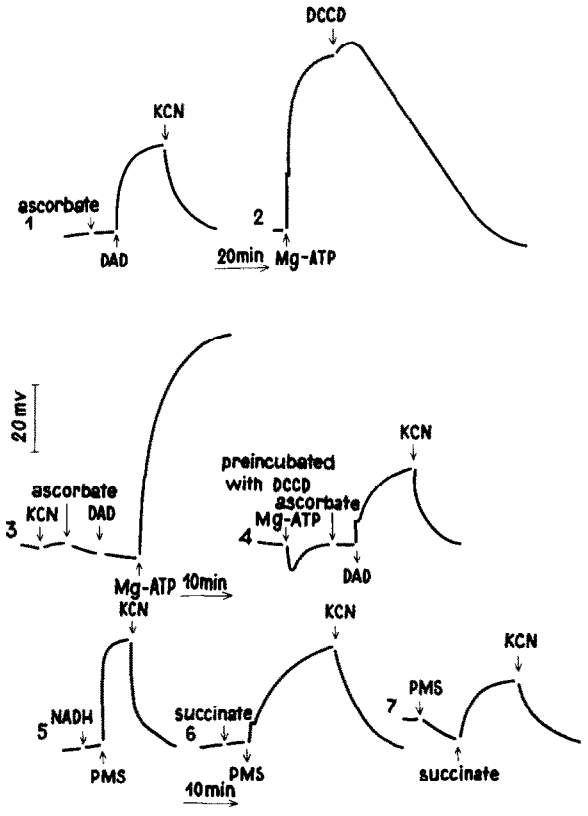

Fig.1. Electrical responses of the filter-associated submitochondrial particles. Incorporation of SMP was carried out as in section 2 for 30 min. The final reaction mixture contained 0.25 M sucrose, 50 mM Tris–HCl (pH 7.7) and 10 mM $(NH_4)_2SO_4$. Other additions: ascorbate, 5 mM; KCN, 2 mM; DAD, 0.2 mM; Mg-ATP, 2 mM; DCCD, 4×10^{-4} M (trace 2), or 40 min preincubation with 2×10^{-4} M (trace 4); NADH, 1 mM; PMS, 80 μM; succinate, 5 mM.

Mitex filters was typically 1–5 min, which is notably faster than in [22] with a different porous teflon film (cf. [23]). The magnitude of the electrogenic activity varied somewhat depending on the preparation; the maximal values of the responses were ~80 mV both with ATPase and cytochrome oxidase, although the former was usually more active electrically than the latter.

Uncouplers are used traditionally in order to show the dependence of a certain process on membrane-linked energy transduction. The use of classical protonophorous uncouplers like FCCP is not however justified in case of the filter-associated membranous vesicles (cf. [23,24]) since the electrical resistance of the filter is severely decreased by these agents. On

the other hand the coupling membrane of the filter-associated vesicles can be short-circuited selectively, without a concomitant major increase in conductivity of the filter, by a non-diffusible ionic-channel type ionophore, e.g., gramicidin A. We have observed that gramicidin added to the SMP-containing compartment of the chamber abolished and prevented the electrical responses linked to the ATPase and the cytochrome oxidase activities of SMP provided that KCl was present in the reaction mixture. In the absence of KCl, or when added to the SMP-free compartment, gramicidin inhibited the electrogenic activity only slightly.

The sensitivity of the electrogenic activity of the filter-associated SMP to specific inhibitors of the corresponding enzymes and to gramicidin A proves the electrical responses observed to originate in $\Delta\psi$ across the coupling membrane of submitochondrial particles. Note that $(NH_4)_2SO_4$ was present in the reaction mixture, which precluded ΔpH formation.

The addition of NADH or succinate (fig.1, traces 5 and 6, respectively) does not result in electrical responses of the filter-associated SMP. Under these conditions the electrogenic activity can be conferred on the particles by the redox mediator phenazine methosulfate (PMS) which can accept electrons from dehydrogenases and feed them into the respiratory chain at the level of cytochromes $c + c_1$, by-passing the CoQ–cytochrome *b* segment of the redox chain. That the PMS-induced responses arise from the cytochrome oxidase activity is indicated by the inhibitory action of cyanide.

In accordance with [22], no electrogenic activity of any reaction in the respiratory chain that involves CoQ could be detected with the technique used. The NADH-ferricyanide, NADH-duroquinone, and NADH-fumarate reductase activities, as well as the NADH oxidase, succinate oxidase and duroquinol oxidase reactions were silent (at least electrically) in the filter-associated SMP (not shown, [22]). Presumably these redox activities are supressed upon incorporation of the particles into the filter. The same type of inhibition of the cyclic electron flow at the level of CoQ has been observed in this group in experiments with *Rhodospirillum rubrum* chromatophores attached to thick planar membranes or lipid-impregnated filters [21].

A suggestion [21] that CoQ is extracted from the membrane of the associated particles into a hydrophobic phase of the filter, although per se meaningful, is not sufficient to explain the impairment of the CoQ-linked functions in the filterassociated particles since the inclusion of CoQ_6 into the lipid mixture impregnating the filter did not restore any of the missing CoQ-dependent activities. Therefore we tentatively suggest that it is the hydrocarbon solvent in which the filter-impregnating phospholipids are dissolved that inhibits the respiratory chain of SMP in the domains responsible for CoQ binding; that these sites are susceptible to non-specific inhibition by a large number of hydrophobic compounds has been shown [26]. In particular hydrocarbon solvents, such as isooctane, impair the electron transfer in SMP at the level of CoQ [27].

In [22] we applied the procedure developed for chromatophores of photosynthetic bacteria [21], viz. a prolonged incubation (several hours) in the presence of high concentrations of bivalent cations to incorporate SMP into the lipid impregnated filters. With Mitex filters, we were able to greatly simplify the procedure. As shown in fig.2A the ATP-linked response can already be observed within 2 min after addition of SMP to one of the filter-separated compartments of the measuring cell. In 5 min the electrogenic activ-

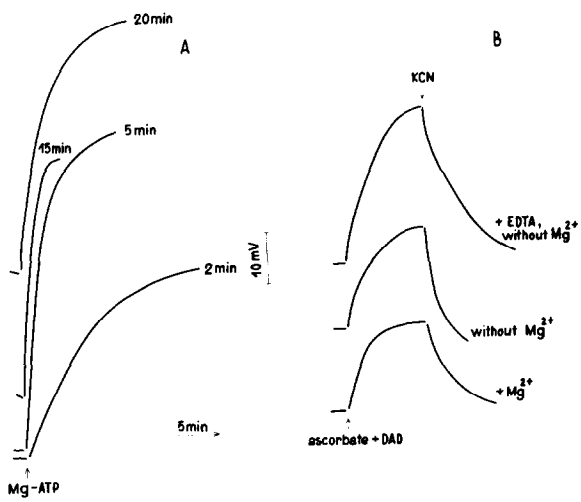

Fig.2. Electrogenic activity in the SMP–phospholipid-impregnated filter system under various conditions of the association procedure. (A) SMP were associated with the filter as in section 2 but varying the preincubation time as indicated; Mg-ATP, 2 mM. (B) Association of SMP with the filter was carried out for 20 min in the presence of 30 mM $MgSO_4$, in the absence of divalent cations added, and in the presence of 1 mM EDTA to deplete endogenous Mg^{2+}. Other conditions were as in fig.1.

ity virtually saturates in amplitude and in rate of the response. A very rapid development of the measurable photoelectric activity could also be observed with the Mitex filters and *Rhodospirillum rubrum* chromatophores (not shown).

Fig.2B shows that as soon as the Mitex filters are used for SMP incorporation, there is no longer a specific requirement for bivalent cations. The association of SMP with the filter can be achieved in the absence of added Mg^{2+} and even in the presence of EDTA, as displayed by the electrogenic activity of cytochrome oxidase. Similar results have also been obtained with ATPase-linked electrical responses (not shown, [24]), although in this case some amount of Mg^{2+} had to be added as the substrate (Mg-ATP).

4. Concluding remarks

Association of SMP with lipid-impregnated teflon filters appears to be a very simple way to assay the electrogenic function of the cytochrome oxidase, ATPase and transhydrogenase activities [22]. The following advantages of this approach can be suggested:

(i) It is possible to apply an external electric field across the filter associated with SMP and thus to study the kinetic and thermodynamic relationships between $\Delta\psi$, of the desired polarity and magnitude, and the electrogenic and, hopefully, the enzymatic activities of the mitochondrial proton pumps. Although very sensitive methods of enzymatic activity measurements would have to be used in the latter case, due to the small amount of SMP that adheres to the filter, the results in [24] are encouraging.

(ii) Time resolution of the electrogenic activity measurements with the filter-associated membranous systems is $\sim 10^{-4}$ s and can be easily adjusted to $\sim 10^{-7}$ s, if the teflon filters are replaced by the collodion films [15–18]. Thus the method may provide an as yet unique opportunity to investigate the rapid kinetics of the cytochrome oxidase electrogenic function if combined with carbon monoxide/flash photolysis techniques [28,29].

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