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Characterization and reconstitution of the Na^+/H^+ antiporter from the plasma membrane of the halotolerant alga *Dunaliella*

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Na^+/H^+ exchange activity in plasma membrane preparations isolated from the unicellular halotolerant alga, *Dunaliella salina*, is shown to be competitively inhibited by amiloride or Li^+, with K_i values of 25 and 30 μM, respectively. The activity can be followed by either the sodium-dependent change in transvesicular ΔpH, as monitored by absorbance changes of Acridine orange, or by the ΔpH-dependent uptake of $^{22}Na^+$ into the intravesicular space. The activity was solubilized, by extraction with Triton, and reconstituted into active proteoliposomes. The activity of the reconstituted proteoliposomes was strongly stimulated by the presence of valinomycin and KCl, suggesting that the exchanger is electrogenic, presumably exchanging more than one proton for each Na^+ ion. Partial purification of the Triton-extracted exchanger was obtained by fractionation on a DEAE-cellulose column.

Introduction

Na^+/H^+ antiporters have been described in many bacterial and animal plasma membranes, and postulated to play a major role in intracellular pH and Na^+ concentration regulation [1,2]. There are only a few reports on a Na^+/H^+ antiporter within isolated plant membranes, mostly within the tonoplast which separates the relatively Na^+-rich and acidic vacuole from the Na^+-poor and neutral cytoplasm [3,4]. We recently described the identification of a Na^+/H^+ antiporter within plasma membrane preparations isolated from the halotolerant alga *Dunaliella* [5]. The exchanger was highly specific to Na^+, and inhibited by Li^+ and amiloride. It was suggested that the exchanger may play a role in the maintenance of the low intracellular Na^+ (below 100 mM) in these algae when grown in media containing 0.5 to 4 M NaCl [6–8].

Despite the extensive available knowledge on the properties and function of the animal and bacterial exchanger, only a few successful attempts were reported of solubilization, purification and reconstitution of the Na^+/H^+ antiport protein [9–12].

We report herein on the further characterization, solubilization, partial purification and reconstitution of the Na^+/H^+ exchanger of isolated plasma membranes from *Dunaliella*.

Materials and Methods

Preparation of plasma membrane. Plasma membranes were prepared as previously described [5]. Before solubilization the plasma membranes were further purified by centrifugation through a 5–50% glycerol gradient for 90 min at 250000 × g. The pellet was resuspended in 0.4 M glycerol, 10 mM KCl, 2 mM $MgCl_2$, 10 mM Tris-Mops (pH 7.0) and hand homogenized.

Electron microscopy. Electron microscopy of purified plasma membrane vesicles was performed as previously described [13].

Solubilization. To 0.25 ml of purified plasma membranes (containing 1 mg protein) in a medium containing 2 M glycerol, 1 mM DTT, 2 mM $MgCl_2$, 10 mM Tris-Mops (pH 7.0) were added Triton X-100 to 0.8% and 0.4 mg phospholipids (prepared as described). Incubation was for 15 min at 0 °C followed by centrifugation for 10 min at 150000 × g. The supernatant was used for reconstitution.

Preparation of phospholipids. 30 mg of soybean phospholipids (Sigma phosphatidylcholine Type II-S) and 10 mg cholesterol were solubilized in chloroform. The chloroform was evaporated by flushing with nitrogen, diethyl ether was added and a dry lipid film was formed by further flushing with nitrogen. 1 ml of 50 mM KCl,

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0.1 mM EDTA, 1 mM DTT, 10 mM Tris-Mops (pH 7.0) was added, the lipids mixed vigorously for 10 min, 1.2% cholate added and the mixture sonicated in a bath-type sonicator to almost clarity. To remove the cholate and form liposomes, the mixture was then passed through a 10 ml Sephadex G-50 coarse column equilibrated and eluted with 0.25 M sucrose, 2 mM $MgCl_2$ and 10 mM Tris-Mops (pH 7.0). About 1–1.5 ml of the turbid eluent was collected.

Reconstitution. One volume of the Triton-soluble extract was mixed with two volumes of liposomes. After incubation for 30 min at 0 °C the mixture was dialyzed overnight against 0.25 M sucrose, 2 mM $MgCl_2$, 10 mM Tris-Mops (pH 7.0) and SM-4 Bio-Beads that absorb Triton, and kept under liquid nitrogen. The proteoliposomes can be frozen and thawed several times, with no decrease in activity.

Purification. The Triton-soluble extract (3 ml) was loaded on a 10 ml column of DEAE-cellulose (Fractogel-TSK, DEAE-650) that was preequilibrated with 20% Glycerol, 0.5 mM $MgCl_2$, 0.2% Triton, 1 mM DTT, 0.01% phospholipids, 2.5 mM Tris-Mops (pH 7.0). The column was washed with 20 ml of the same medium followed by a KCl gradient 0–150 mM in the same medium. Fractions were reconstituted and assayed as described.

Measurement of Na^+/H^+ exchange activity. (1) Acridine orange assay. This assay monitors the changes in transvesicular ΔpH and was employed essentially as described [5], except for the base used to create the initial transmembrane ΔpH which was tetramethyl ammonium hydroxide (TMA-OH). With proteoliposomes only 1 min preincubation in acid was employed.

(2) $^{22}Na^+$ uptake assay. Membrane vesicles or proteoliposomes containing about 0.4 mg protein were

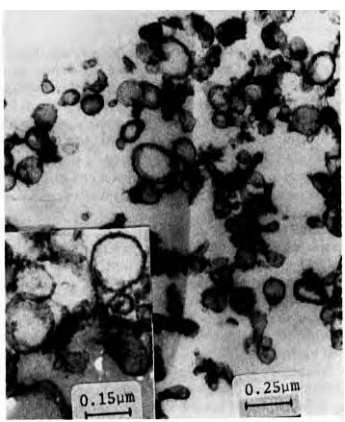

Fig. 1. Electron micrographs of an isolated plasma membrane preparation. Purified plasma membranes were prepared and analysed as described under Materials and Methods.

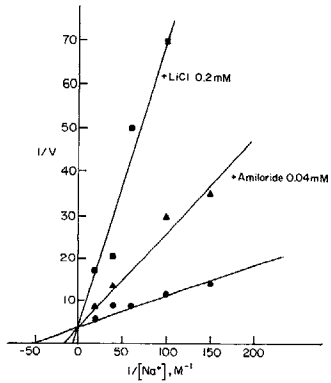

Fig. 2. Competitive inhibition by amiloride or Li^+ of the Na^+ dependent H^+ flux in plasma membrane vesicles. Na^+/H^+ exchange was measured with the Acridine orange assay as described under Materials and Methods.

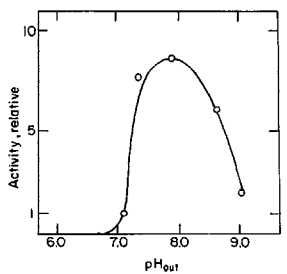

Fig. 3. Effect of external pH on Na^+ dependent H^+ flux. Na^+/H^+ exchange was measured with the Acridine orange assay, except for the amount of TMA-OH added which was varied to yield the indicated external pH. Each point represents the difference between the rate of H^+ flux in the presence of Na^+ and the rate in its absence.

preincubated with 20 mM succinate (pH 5.2) for 10 min or 1 min, respectively, at 10 °C. A mixture containing premeasured amount of TMA-OH to reach a medium pH of 8.0 and 300 μM $^{22}Na^+$ was added, followed by incubation for 5 min at 10 °C and application to a 3 ml column of Dowex 50W-X8, (50–100 mesh) which was preequilibrated with Tris and prewashed with water [7]. Elution was with 2 ml 0.4 M glycerol, 5 mM Tris-Mops (pH 7.0) for the plasma membrane and 0.25 M sucrose, 5 mM Tris-Mops (pH 7.0) for the proteoliposomes. The eluent was added to scintillation fluid and the radioactivity determined in a scintillation counter.

Protein determination. Protein was measured by a modification of Lowry method [14]. Protein in the fractions eluted from the DEAE column was assayed with fluorescamine [15].

Polypeptide composition. The polypeptide composition of the membranes was determined by SDS-PAGE following Laemmli with a 7%–15% gradient [16].

Results

Properties

The plasma membrane fraction used in most of these studies is shown in Fig. 1. It consists of a heterogeneous mixture of mostly unilamellar vesicles with a diameter between 50 and 250 nm. Most of the vesicles appear devoid of any internal structure.

It was previously reported [5] that the Na^+/H^+ exchange activity of this fraction was inhibited by Li^+ and amiloride. Fig. 2 shows a Lineweaver-Burk plot of these inhibitions, indicating that both are apparently competitive with Na^+. The apparent K_i for Li^+ is 30 μM, and for amiloride, 25 μM. The K_m for Na^+ was similar to that previously reported, around 20 mM [5].

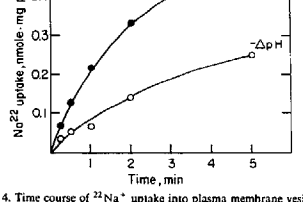

Fig. 4. Time course of $^{22}Na^+$ uptake into plasma membrane vesicles. Na^+/H^+ exchange was measured by the $^{22}Na^+$ uptake assay as described under Materials and Methods. The data marked $-\Delta pH$ were obtained by omitting succinate from the acidic incubation and replacing TMA-OH by TMA-Cl in the basic mixture.

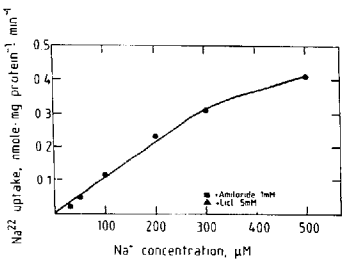

Fig. 5. Effect of external sodium concentration on $^{22}Na^+$ uptake into plasma membrane vesicles. Assays as described under Fig. 4, except for the NaCl concentration which was varied as indicated. Also indicated are the effects of 1 mM amiloride or 5 mM LiCl in the presence of 300 μM NaCl.

The effect of external pH on the rate of the Na^+/H^+ exchange activity is shown in Fig. 3. Optimal activity occurred within a narrow range of external pH 7.8–8.3. This may be due to a rather strong dependence on external pH, or to a dependence on ΔpH coupled with an inhibition by external pH exceeding 8, since in this experiment both external pH and ΔpH are simultaneously varied.

Na^+/H^+ exchange could also be measured by following the uptake of $^{22}Na^+$ as described under Methods. As can be seen in Fig. 4 a substantial uptake was observed also in the absence of an imposed ΔpH. This was subtracted in all subsequent experiments which report the ΔpH induced $^{22}Na^+$ uptake.

Fig. 5 illustrates the dependence of $^{22}Na^+$ uptake on the external $^{22}Na^+$ concentration. Clearly the rate of Na^+ uptake was linear up to around 300 μM Na^+ and was inhibited by Li^+ and amiloride, as found with the Acidine orange assay.

Solubilization, purification and reconstitution

To assay a solubilized exchanger, it is necessary to first reconstitute it into a relatively impermeable liposome. It was found that such tight proteoliposomes could be prepared by employing a mixture of soybean phospholipids and cholesterol at a ratio of 3 : 1. As can be seen in Fig. 6 such proteoliposomes are less permeable to protons than the native plasma membrane vesicles. Proteoliposomes made from soybean phospholipids alone or *E. coli* phospholipids were found to exhibit considerable leakage to protons which made this type of assay unsuitable.

Fig. 6 shows that proteoliposomes, prepared as described under Methods, exhibited easily measurable Na^+/H^+ exchange activity indicating that the protein

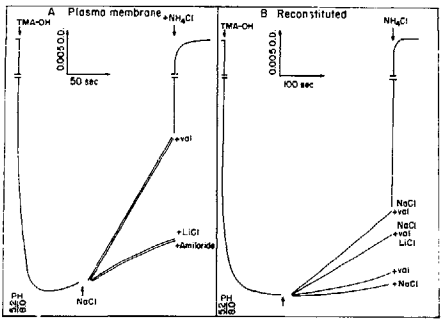

Fig. 6. Na^+/H^+ exchange activity in plasma membrane vesicles and in reconstituted proteoliposomes. Na^+/H^+ exchange was measured with the Acridine orange assay as described under Materials and Methods. The plasma membrane preparation contained 80 μg protein and the proteoliposomes 45 μg protein. Additions: NaCl, 50 mM; LiCl, 50 mM; amiloride, 0.4 mM; NH_4Cl, 25 mM; val refers to addition of valinomycin, 0.4 μM and KCl, 30 mM.

was indeed solubilized and reconstituted. However, the activity in the proteoliposomes did not exhibit identical properties to that in the native plasma membrane. It was less inhibited by Li^+, almost insensitive to amiloride, and highly stimulated by the presence of valinomycin $+K^+$. It was previously shown [5] that the Na^+/H^+ activity of the plasma membrane vesicles did not respond to the presence of valinomycin $+K^+$, which was consistent with the notion that the exchanger may be electroneutral. Since the solubilized and reconstituted exchanger was highly stimulated by valinomycin $+K^+$ (Fig. 6) it seems most reasonable to conclude that the exchanger is not electroneutral in nature, but that the native plasma membrane is sufficiently permeable to ions so as to make imperceptible the effects of the added permeability to K^+ induced by valinomycin.

The successful solubilization and reconstitution of the Na^+/H^+ exchange made it possible to attempt to purify the solubilized antiporter. Fig. 7 illustrates the fractionation of the solubilized exchanger on a DEAE-cellulose column. As can be seen the majority of the antiporter activity was eluted just following the void volume, while most of the protein eluted much later (following the addition of 0.3 M KCl). This is further illustrated in the SDS-PAGE patterns of the various fractions (Fig. 8). Lane 1 represents the plasma membrane preparation, and lane 2 the same preparation following the glycerol gradient centrifugation (see Methods). Lane 3 contains the solubilized plasma membrane added to the column, and lane 4 a concentrate of the purified fractions eluted from the column following the void volume. Lane 5 contains a similar purified fraction, stained with silver rather than Coomassie blue. Several bands are still present but the preparation has clearly been highly purified.

Attempts at further purification proved difficult due to loss of activity of the purified fractions.

Discussion

The Na^+/H^+ antiporter of the plasma membrane of *Dunaliella* is shown here to be competitively inhibited by Li^+ and amiloride at relatively low concentrations.

The antiporter was completely solubilized with Triton and actively reconstituted into artificial phospholipid/cholesterol liposomes. Solubilization was also effective with cholate (not shown). One of the major difficulties in assaying the reconstituted activity was due to the relative proton permeability of reconstituted phospholipid liposomes. It was found that addition of cholesterol to soybean phospholipids in a ratio of 1:3 produced rather proton impermeable liposomes which were still active in reconstitution.

The reconstituted antiporter was much less sensitive to inhibitions by amiloride or Li^+. This may relate to its mode of association with the artificial phospholipid/cholesterol liposome, or to the relative solubility of the inhibitors in the new matrix. On the other hand, it was highly stimulated by valinomycin $+K^+$. It seems most reasonable to assume that this represents the true electrogenic nature of the exchanger. The fact that this effect is not observed in the native plasma-membrane vesicles may be due to sufficient permeability to some other ionic species through the native membrane. If the

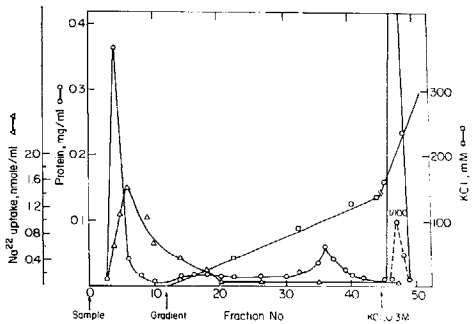

Fig. 7. Elution profile of solubilized plasma membranes on a DEAE-cellulose column. Triton-solubilized plasma membranes (3 ml containing 12 mg protein) were loaded on a 10 ml column of DEAE-cellulose and eluted as described under Methods.

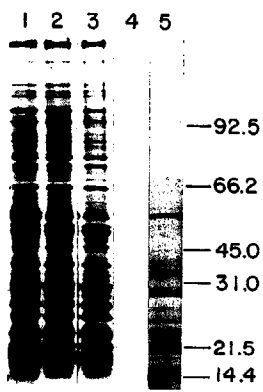

Fig. 8. SDS-PAGE of plasma membranes and purified fractions. Fractions were concentrated with trichloroacetic acid and deoxycholate as previously described [14] and fractionated on a 7.5–15% SDS gradient gel [16]. Lane 1, crude plasma membrane, stained with Coomassie blue; lane 2, purified plasma membrane, stained with Coomassie blue; lane 3, Triton-solubilized plasma membrane, stained with Coomassie blue; lane 4, active fractions from DEAE-cellulose column, stained with Coomassie blue; lane 5, As lane 4, but stained with silver [17].

exchanger has a function in eliminating intracellular Na^+, it is indeed most likely to be electrogenic, moving more than one proton for each Na^+. In these algae the ratio of extracellular to intracellular Na^+ concentrations can exceed 100 [7], and it is therefore unlikely to fulfill a significant function in this respect if driven solely by the small pH gradient which may be expected to be present across the plasma membrane.

The purification achieved thus far, although significant, is insufficient to identify the protein responsible for the exchange activity. A significant improvement in the stabilization of the isolated protein will be required before further purification and identification can be successfully attempted.

References

1 Krulwich, T.A. (1983) Biochim. Biophys. Acta 726, 245–264.
2 Aronson, P.S. (1985) Annu. Rev. Physiol. 47, 545–560.
3 Blumwald, E. and Poole, R.J. (1985) Plant Physiol. 78, 163–167.
4 Blumwald, E. and Poole, R.J. (1987) Plant Physiol. 83, 884–887.
5 Katz, A., Kaback, H.R. and Avron, M. (1986) FEBS Lett. 202, 141–144.
6 Katz, A. and Avron, M. (1985) Plant Physiol. 78, 817–820.
7 Pick, U., Karni, L. and Avron, M. (1986) Plant Physiol. 81, 92–96.
8 Bental, M., Degani, H. and Avron, M. (1988) Plant Physiol. 87, 813–817.
9 Friedrich, T., Sablotni, I. and Burckhardt, G. (1986) J. Membr. Biol. 94, 253–266.
10 LaBelle, E.F. and Lee, S.O. (1982) Biochemistry 21, 6293–2697.
11 Nakamura, T., Hsu, C. and Rosen, B.P. (1986) J. Biol. Chem. 261, 618–683.
12 Weinman, E.J., Shenolikar, S., Cragoe, E.J. and Dubinsky, W.B. (1988) J. Membr. Biol. 101, 1–9.

13 Ben-Amotz, A., Katz, A. and Avron, M. (1982) J. Phycol. 18, 529–537.
14 Bensadoun, A. and Weinstein, D. (1976) Anal. Biochem. 70, 241–250.
15 Harris, D.A. (1987) in Spectrophotometry and Spectrofluorimetry (Harris, D.A. and Bashford, C.L., eds.), pp. 63–64, IRL press, Oxford.
16 Laemmli, V. (1970) Nature 227, 680–685.
17 Wray, W., Bonlikas, T., Wray, V.P. and Hancock, R. (1981) Anal. Biochem. 118, 197–203.