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Reconstitution of a chloride-translocating ATPase from *Aplysia californica* gut

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Basolateral membranes of *Aplysia* foregut epithelia contain both a Cl^--stimulated ATPase activity and an ATP-dependent Cl^- transport. The protein responsible for both of these biochemical activities (Cl^- pump) can be solubilized and reconstituted into liposomes with the aid of the detergent digitonin. Proteoliposomal Cl^- pump activity was inhibited by vanadate.

There is no direct, rigorous proof of primary active Cl^- transport in animal plasma membranes; that is, an ATPase that translocates Cl^- up its electrochemical potential gradient powered by the simultaneous hydrolysis of ATP. In fact, the evidence for a primary active Cl^- transport mechanism in plants [1,2] and bacteria [3] is more convincing than what has been demonstrated in animal plasma membranes [4,5]. It has been hypothesized that Cl^- transport across the *Aplysia californica* foregut is mediated by a primary active transport process [6]. Lending credence to this idea were the demonstrations of: (1) the existence of Cl^--stimulated ATPase activity in *Aplysia* foregut absorptive cell basolateral membranes (BLM) [7], and (2) that there was an electrogenic ATP-dependent Cl^- uptake in these same *Aplysia* foregut absorptive cell BLM vesicles [8,9]. In the present study, I have included the essence of a reconstitution procedure described by McCormick et al. [10] which demonstrates incorporation of Cl^- stimulated ATPase activity into liposomes that yield enrichment of a concomitant primary active Cl^- transport activity (Cl^- pump).

Seahares (*Aplysia californica*) were obtained from Marinus (Westchester, CA) and were maintained at 25 °C in circulating filtered seawater. Adult *Aplysia* (400–1000 g) were used in these experiments. The inside-out BLM vesicles were prepared from *Aplysia* foregut epithelial cells by homogenization and differential and discontinuous sucrose density-gradient centrifugation techniques as described previously [7,9]. Briefly, the membrane layer at the 40–50% sucrose interface was removed (BLM), suspended in a choline chloride medium (10 mM imidazole-HCl (pH 7.8), 250 mM sucrose, 3 mM $MgSO_4$ and 25 mM choline chloride) and centrifuged at 200 000 × *g* for 60 min to obtain a pellet that was then homogenized in a choline chloride medium (above) with a glass-Teflon homogenizer and recentrifuged at 200 000 × *g* for 60 min. The final membrane pellet was suspended in the same buffer at 2 mg protein/ml. Cl^--stimulated ATPase (EC 3.6.1.3) was measured in a reaction mixture as also previously described [7], as was the ATP-dependent Cl^- transport in the BLM vesicles [8,9]. Released inorganic phosphate (P_i) was estimated from the protein-depleted supernatant by the method of Fiske and SubbaRow [11]. Alkaline phosphatase (EC 3.1.3.1) and Na^+/K^+-ATPase were assayed as described by Lee and Pritchard [12]. Cytochrome *c* oxidase was measured according to the method of Cooperstein and Lazarow [13]. (Ca^{2+} + Mg^{2+})-ATPase was assayed as described by Van Amelsvoort et al. [14]. Protein concentrations (regular and microassay) were determined using the Bio-Rad reagent (Bio-Rad, Richmond, CA). Differences between means were analyzed statistically using Student's *t*-test with a $P < 0.05$ used as a statistical difference criterion.

Cl^- pump activity was solubilized by a modification of the method of McCormick et al. [10]. BLM were diluted (1 : 2, v/v) with solubilization buffers (10 mM imidazole-HCl (pH 7.8), 250 mM sucrose, 3 mM $MgSO_4$, 25 mM choline chloride, 1 mM Tris ATP, 1 μM phenylmethylsulfonyl fluoride, and either of the following detergents: 2% sodium cholate, 1.5% Lubrol PX, 2% octyl glucoside, or 2% digitonin) to give a final protein concentration of about 3 mg/ml. After incubation for

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35 min at 4°C, the insoluble membrane fragments were removed by centrifugation at 100 000 × *g* for 60 min.

Moist copolymer beads (0.3 g) (BioBeads SM-2: Bio-Rad, Richmond, CA) were added to 1.2 ml protein samples containing one of the following detergents: sodium cholate, lubrol PX, octyl glucoside or digitonin, to remove the detergents by binding. Samples were then gently agitated at 4°C on a blood tube rotator for 45 min. Supernatants from each sample were withdrawn manually via syringe and needle at 4°C.

The basic reconstitution protocol was adapted from that described by McCormick et al. [10] for their studies on transport in the Ehrlich ascites cell membrane. Reconstitution of Cl^- pump activity was performed by mixing 0.5 mg of solubilized membrane protein with 10 mg of sonicated asolectin and either 1% sodium cholate, 0.6% Lubrol PX, 1% octyl glucoside, or 1% digitonin in a total volume of about 1 ml. The solution was diluted with 5–10 vol. of imidazole buffer, and then sonicated for 20 s. The proteoliposomes were pelleted by centrifugation at 100 000 × *g* for 60 min and then resuspended in 350 μl of imidazole buffer for immediate use.

Differential centrifugation of the mucosal homogenates followed by discontinuous sucrose density gradient centrifugation produced a plasma membrane fraction located at the 40–50% sucrose interface identified previously as putative BLM [7]. As shown in Table I, ouabain-sensitive Na^+/K^+-ATPase activity (a BLM marker) was enriched approx. 8.0-fold in the same preparation. Cytochrome *c* oxidase (a mitochondrial marker) was below detectable activity levels. Cl^--stimulated ATPase activity was enriched 3.0-fold in the same membrane fraction and all reconstitution experiments reported below were performed using this membrane fraction. These results, coupled with previous observations [7,9], suggest that this membrane fraction is a relatively pure BLM preparation that is free from mitochondrial-contaminant Cl^--stimulated ATPase activity [6].

TABLE I

Distribution of marker enzymes and Cl^--stimulated ATPase activity during preparation of plasma membranes from Aplysia foregut absorptive cells

Conditions for the enzyme assay were as described in Materials and Methods. Values are mean ± S.E. from six different preparations. Enzyme activity is expressed as μmol/h per mg protein for Na^+/K^+-ATPase and alkaline phosphatase; Δ, log [ferrocytochrome *c*]/min per mg protein for cytochrome *c* oxidase; μmol/15 min per mg protein for Mg^{2+}- and Cl^--ATPase. H, homogenate; BLM, 40 to 50% sucrose interface; n.d., not detectable. Starting gut mucosa was approx. 5.0 g.

Enzyme	H	BLM
Na^+/K^+-ATPase	0.92 ± 0.38	7.28 ± 1.85
Alkaline phosphatase	1.06 ± 0.43	0.86 ± 0.30
Cytochrome *c* oxidase	0.72 ± 0.20	n.d.
Mg^{2+}-ATPase	2.15 ± 0.50	8.47 ± 1.39
Cl^--ATPase	2.73 ± 0.61	7.96 ± 1.08

TABLE II

Reconstitution of Cl^- catalytic and transport activities

Values are means ± S.E. from four individual determinations. Enzyme activity is expressed as μmol/15 min per mg protein for Mg^{2+}- and (Mg^{2+} + Cl^-)-ATPase. Conditions for the enzyme assay are described in Materials and Methods. Time period of assay for V_i determined previously [7]. Vanadate (0.1 mM) had no significant effect on Mg^{2+}-ATPase activity. Either vanadate (0.1 mM) was preincubated with the proteoliposomes in the reaction mixture (50 μl containing 10 mM imidazole-HCl, 250 mM sucrose, 3 mM $MgSO_4$, and 25 mM choline chloride) at pH 7.8 for 10 min at 25°C, or 5 mM ATP was added to the reaction mixture to initiate the incubation for the transport experiments. The incubation for the uptake of $^{36}Cl^-$ was measured for 10 min at 25°C. Time for steady-state values for both ATP-independent and ATP-dependent $^{36}Cl^-$ uptakes was based on previous observations [8,9]. $^{36}Cl^-$ uptake is expressed as nmol/mg protein. + Represents a compound's presence in the reaction mixture; − represents its absence; n.d., not detectable.

(A) Proteoliposome ATPase activity

Extractive and reconstitutive detergents	Mg^{2+}-ATPase	(Mg^{2+} + Cl^-)-ATPase	(Mg^{2+} + Cl^-)-ATPase + vanadate
Cholate	n.d.	n.d.	−
Octyl glucoside	n.d.	n.d.	−
Lubrol PX	n.d.	n.d.	−
Digitonin	2.8 ± 0.4	11.2 ± 2.0	2.9 ± 0.4

(B) Cl^- uptake into proteoliposomes

Extractive and reconstitutive detergents	− ATP	+ ATP	+ ATP + vanadate
Cholate	85.7 ± 5.6	82.7 ± 6.9	80.6 ± 8.3
Octyl glucoside	82.7 ± 8.0	73.6 ± 9.2	83.9 ± 8.9
Lubrol PX	28.3 ± 11.1	39.9 ± 13.9	39.3 ± 14.0
Digitonin	91.2 ± 6.0	192.5 ± 9.3	93.1 ± 7.9

As shown in Table II, Cl^--stimulated ATPase activity exists significantly ($P < 0.05$) above Mg^{2+}-stimulated ATPase activity found in the proteoliposome population extracted and generated with digitonin. Vanadate (0.1 mM) inhibited this Cl^--stimulated ATPase activity by 99%. From this digitonin-generated proteoliposome population, it is also seen in Table II that there is a significant ATP-dependent Cl^- uptake into these proteoliposomes above that of control ($P < 0.05$) and that this ATP-dependent Cl^- uptake is also inhibited by 0.1 mM vanadate. Not detected in the proteoliposomes solubilized and formed by digitonin were Na^+/K^+-ATPase, alkaline phosphatase, (Ca^{2+} + Mg^{2+})-ATPase or cytochrome *c* oxidase activities and, coupled with a previous observation [9] that FCCP had no effect on ATP-driven Cl^- accumulation in the BLM vesicles, suggests that none of these enzymes nor eukaryotic H^+-ATPases could express Cl^- pump activity [4]. These data also suggest that these two major observations are manifestations of one molecular mech-

anism: the Cl^- pump. Support of this contention rests with the findings that vanadate (an inhibitor of E_1-E_2-ATPases [15]) inhibited both Cl^--stimulated ATPase activity and ATP-dependent Cl^- transport in the digitonin-based proteoliposomes (Table II). These results with vanadate are similar to what has been observed in the BLM of *Aplysia* foregut [7,9].

Na^+ cholate, Lubrol PX or octyl glucoside, acting in both solubilization and reconstitution protocols, respectively, did not incorporate viable Cl^- pumps into their respective liposome populations. This is seen in Table II, where no Cl^--stimulated ATPase activity or ATP-dependent Cl^- transport was observed when any of these detergents was used in these protocols.

This study provides evidence for both a rapid and efficient reconstitution procedure for Cl^- pump activity of invertebrate gut tissue, besides providing evidence that all components of the solubilized ATPase have been extracted intact.

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