Characterization of the ATP-dependent Proton Pump of Clathrin-coated Vesicles

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The ATP-dependent proton pump which was previously identified in clathrin-coated vesicles isolated from calf brain (Forgac, M., Cantley, L., Wiedemann, B., Altstiel, L., and Branton, D. (1983) Proc. Natl. Acad. Sci. U.S.A. 80, 1300–1303) is further characterized. 7-Chloro-4-nitrobenz-2-oxa-1,3-diazole (NBD-Cl) was identified as a potent inhibitor of both ATP-dependent proton uptake and Mg\textsuperscript{2+}-ATPase activity of coated vesicles. Thus, incubation with 10 μM NBD-Cl for 10 min at 23 °C caused the loss of 80% of the Mg\textsuperscript{2+}-ATPase activity and 95% of the proton pumping activity. The observed protection from NBD-Cl inhibition by ATP suggests that NBD-Cl may react at the catalytic site, and reversal of NBD-Cl inhibition by 2-mercaptoethanol is consistent with reaction at either a tyrosine or cysteine residue.

Various lines of evidence have suggested that exposure to low pH is the signal which activates ligand-receptor dissociation following receptor-mediated endocytosis (1–6), and a number of studies have indicated that this acidification occurs in a prelysosomal compartment (6–9). We recently reported that clathrin-coated vesicles contain an ATP-dependent proton pump capable of acidifying the vesicle interior (10) and suggested that this pump is responsible for the acidification event required for ligand-receptor dissociation during receptor recycling back to the cell surface. A similar result, again in brain-coated vesicles, has also been reported by Stone et al. (11). In addition, evidence has been obtained for the existence of an ATP-dependent proton pump with essentially identical properties in endosomes derived from a number of cell types (12, 13). In the present communication, we provide a more detailed characterization of the ATP-dependent proton pumping activity of clathrin-coated vesicles.

MATERIALS AND METHODS

Clathrin-coated vesicles were prepared from calf brain by the procedure of Wiedemann and Minna (14). As with the procedure previously employed (10), electron microscopy indicated that >95% of the vesicles were coated (with virtually all of the clathrin-coated structures containing a membrane vesicle). Vesicles were stored in 100 mM MES (pH 6.5), 0.5 mM MgCl\textsubscript{2}, 1.0 mM EGTA, 0.02% sodium azide at 4 °C and were used within 2 weeks of preparation.

Proton transport using the [35S]methylenetramine trapping procedure and ATPase activity were measured as previously described (14). All experiments were carried out in 60 mM KCl, 10 mM NaCl, 10 mM HEPES (pH 7.5), 0.10 mM EGTA at 23 °C unless otherwise indicated. Proton transport activity is expressed as the difference between the [35S]methylenetramine trapped after 2 min in the presence of 1.0 mM ATP (triameterate salt), 2.0 mM MgSO\textsubscript{4}, and that trapped in the presence of 3.0 mM KCl, 2.0 mM MgSO\textsubscript{4}. Typically, addition of ATP resulted in the generation of a 5–7-fold increase in the amount of [35S]methylenetramine trapped. Mg\textsuperscript{2+}-ATPase activity is defined as the fraction of the total ATPase activity that is resistant to 100 μM strophanthidin (typically 70–80%). The specific activity of the Mg\textsuperscript{2+}-ATPase ranged from 0.015 to 0.025 μmol of ATP/min/mg of protein at 23 °C. Relative activities represent the activity observed at a given inhibitor concentration relative to a control which received an equal concentration of solvent (1.0% ethanol unless otherwise indicated). 22Na\textsuperscript{+} and 86Rb\textsuperscript{+} trapping by coated vesicles were measured using a 10-mL Sephadex G-50 column as previously described (16). Protein concentrations were determined by the method of Lowry et al. (16) in the presence of 1.0% sodium dodecyl sulfate.

Phosphorylation by [γ-32P]ATP was carried out as follows. Coated vesicles were stripped of their clathrin coats by incubation in 5.0 mM Tris (pH 8.5), 150 mM sucrose, 0.10 mM EGTA at 23 °C for 1 h (17), followed by centrifugation of the stripped vesicles for 1 h at 100,000 × g. This procedure resulted in loss of approximately 40% of the Mg\textsuperscript{2+}-ATPase activity, 60% of the proton transport activity, and 80% of the protein and was employed to reduce the amount of labeling due to an endogenous protein kinase activity associated with the coated vesicles (18). Essentially identical results were obtained using intact coated vesicles. Stripped vesicles were resuspended to a protein concentration of approximately 2.0 mg of protein/ml in 70 mM KCl, 10 mM HEPES (pH 7.5), 0.10 mM EGTA. Alternatively, samples were prepared which contained an equivalent amount (in ATPase activity) of purified canine kidney (Na\textsuperscript{+},K\textsuperscript{+})-ATPase\textsuperscript{2} in 70 mM NaCl, 10 mM HEPES (pH 7.5), 0.10 mM EGTA (EGTA was used instead of NaCl with the coated vesicles to prevent phosphorylation of any endogenous (Na\textsuperscript{+},K\textsuperscript{+})-ATPase present). To 50-μl samples were added 2.0 mM MgSO\textsubscript{4} and 100 μM cold ATP (prelabeling with cold ATP was employed to reduce labeling at kinase phosphorylation sites). Following a 30-s incubation, 100 μM [γ-32P]ATP (5 Ci/mmol) was added, and the samples were incubated for 15 s followed by either quenching with 0.90 mL of ice-cold 5% trichloroacetic acid or a 15-s chase with 1.0 mM cold ATP and then quenching with 5% trichloroacetic acid. Samples were spun for 2 min at 10,000 × g in a Beckman microfuge, the supernatants were discarded, and the surface of the

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\textsuperscript{1}The abbreviations used are: MES, 2-(N-morpholino)ethanesulfonic acid; NBD-Cl, 7-chloro-4-nitrobenz-2-oxa-1,3-diazole; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis (β-aminoethyl ether) N,N',N''-tetraacetic acid.

\textsuperscript{2}(Na\textsuperscript{+},K\textsuperscript{+})-ATPase was purified from canine kidney by the procedure of Jorgensen (19) and had a specific activity of approximately 10 μmol of ATP/min/mg of protein at 23 °C.

\textsuperscript{K} is unlikely to activate dephosphorylation of the coated vesicle proton pump (as it does with the (Na\textsuperscript{+},K\textsuperscript{+})-ATPase) since K\textsuperscript{+} is not required for ATPase activity (19) and its (K\textsuperscript{+}) analog is not transported during ATP-dependent proton uptake by the coated vesicles (see below).
pellets were washed with 0.15 M potassium phosphate (pH 7.2). Samples were dissolved in 50 μl of 5% 2-mercaptoethanol, 0.25 M sucrose, 0.10 M potassium phosphate (pH 4), 70 mM hexadecylpyridinium chloride, and 1% pyronin Y. Acid gel electrophoresis was carried out as described by Amory et al. (20). Samples obtained from New England Nuclear, and NBD-C1 was purchased from Eastman.

Phosphorylation experiments with inorganic phosphate were also carried out using stripped vesicles (2.0 mg of protein/ml), except that the reaction was done in the presence of 200 μM 32P2O5 (5 Ci/mmol, neutralized with imidazole), 5 mM MgCl2, 20 mM imidazole HCl (pH 7.5), 0.8 mM EGTA for 1 min at 23 °C. In order to control for phosphorylation of the (Na+,K+)-ATPase present in the coated vesicle preparation, the reaction was also carried out in the presence or absence of 60 mM NaCl (which reduces phosphorylation of the (Na+,K+)-ATPase by 32Pi (22)). Quenching and washing with trichloroacetic acid and acid gel electrophoresis were carried out as described above. [3H]Methylamine, 32NaCl, 32RbCl, 32P2O5 and [γ-32P]ATP were obtained from New England Nuclear, and NBD-C1 was purchased from Eastman.

RESULTS AND DISCUSSION

To assist in the purification and characterization of any ion transport protein, it is clearly of value to have an inhibitor of ion transport. NBD-C1 has been previously observed to inhibit the H+-ATPases of mitochondria (23), chloroplasts (24), and bacteria (25), as well as the plasma membrane (Na+,K+)-ATPase (26). As shown in Fig. 1, NBD-C1 is also a potent inhibitor of proton transport and Mg2+-ATPase activity in clathrin-coated vesicles. Thus, reaction with 10 μM NBD-C1 for 10 min at 23 °C resulted in the loss of 95% of the proton transport activity and 80% of the Mg2+-ATPase activity. Even at 50 μM NBD-C1, a fraction of the Mg2+-ATPase activity (i.e. 15%) remained uninhibited, suggesting that this 15% of the total activity is not associated with proton transport in coated vesicles. The half-time of inhibition of both proton transport and Mg2+-ATPase activity at 10 μM NBD-C1 was approximately 2 min (Fig. 2), as compared with half-times of 2 and 11 min at 100 μM NBD-C1 for the (Na+,K+)-ATPase (26) and the mitochondrial ATPase (23), respectively. As can also be seen from Fig. 2, proton transport and Mg2+-ATPase activity are protected from NBD-C1 inhibition by the presence of 2.5 mM ATP, suggesting that NBD-C1 may be inactivating the enzyme by reaction at the catalytic site.

The effect of reducing agents (i.e. 2-mercaptoethanol) on NBD-C1 inhibition of the coated vesicle Mg2+-ATPase was also studied. As can be seen from Table I, if vesicles which had been reacted with NBD-C1 were then treated with 2% 2-mercaptoethanol for 30 min at 23 °C, a complete reversal of
the inhibition of Mg\(^{2+}\)-ATPase activity due to NBD-Cl was observed. The reversibility of NBD-Cl inhibition by reducing agents suggests that NBD-Cl is causing inhibition of the Mg\(^{2+}\)-ATPase by reaction at a tyrosine phenolic group or a cysteine sulfhydryl group rather than a lysine amino group (26). It is interesting in light of this result to note that the sulfhydryl reagent N-ethylmaleimide has also been observed to inhibit the coated vesicle proton pump (11). Vesicles which were treated only with 2-mercaptoethanol (Table I) showed some stimulation of Mg\(^{2+}\)-ATPase activity relative to control vesicles, possibly due to a partial uncoupling effect of the 2-mercaptoethanol or to a reversal of inhibition of Mg\(^{2+}\)-ATPase activity due to partial oxidation of essential sulfhydryl groups during purification of the coated vesicles. If 1 mM 2-mercaptoethanol was included prior to reaction with NBD-Cl, a partial (but not complete) protection of Mg\(^{2+}\)-ATPase activity was observed (Table I). The ability of NBD-Cl to cause partial inhibition of activity even in the presence of excess reducing agent is similar to its effect on the ATP-dependent proton pump in Golgi-derived vesicles (27).

Experiments were carried out to determine whether a phosphorylated intermediate occurred during ATP hydrolysis by the clathrin-coated vesicle proton pumping ATPase. Vesicles from which the clathrin coat had been stripped (see "Materials and Methods") were prelabeled with cold ATP and then given a 15-s pulse of [\(\gamma\)-\(^{32}\)P]ATP followed by quenching or a 15-s chase with excess cold ATP and then quenching. Prelabeling with cold ATP was carried out to reduce the amount of labeling at kinase phosphorylation sites due to the endogenous protein kinase activity associated with coated vesicles (18). Samples were then analyzed using an acid gel electrophoresis system under conditions in which phosphoaspartate bonds are stable (20). As can be seen from Fig. 3, although the 96,000-dalton phosphorylated intermediate of the (Na\(^{+},K\(^{-}\))-ATPase could be readily observed, no difference before and after the cold ATP chase could be seen with the coated vesicle proton pump, despite the presence of equal levels of ATPase activity in the two cases. A phosphorylated intermediate in the 60,000-dalton region could have gone undetected due to the heavy kinase labeling which occurred in this region of the gel. However, it should be noted that all cation pumps previously characterized which form a phosphorylated intermediate have catastrophic subunits with molecular masses of approximately 95,000 daltons or higher (28–32). No rapidly chaseable label was detected in this higher molecular weight range. Alternatively, the phosphorylated intermediate of the coated vesicle proton pump may be appreciably less stable than that of the (Na\(^{+},K\(^{-}\))-ATPase. However, the technique described here has been shown to be capable of detecting the phosphorylated intermediates of not only the (Na\(^{+},K\(^{-}\))-ATPase (33, 34) but also the Ca\(^{2+}\)-ATPase (34), the gastric (H\(^{+},K\(^{-}\))-ATPase (30), and the plasma membrane H\(^{+}\)-ATPases from Neurospora (31) and yeast (32).

We have also attempted to detect a phosphorylated intermediate of the coated vesicle proton pump using \(^{32}\)P, in the presence of Mg\(^{2+}\). This phosphorylation reaction has been observed for both the (Na\(^{+},K\(^{-}\))-ATPase (22) and the Ca\(^{2+}\)-ATPase (35). In the case of the (Na\(^{+},K\(^{-}\))-ATPase, phosphorylation by \(^{32}\)P, is increased by cardiac glycosides (21) and is inhibited by the presence of Na\(^{+}\) (which shifts the enzyme to the E\(_{1}\) conformation (22)). As can be seen in Fig. 4, the only phosphorylated intermediate observed with coated vesicles has properties identical to those associated with the (Na\(^{+},K\(^{-}\))-ATPase (i.e. phosphorylation is enhanced with strophant
din and reduced with Na\(^{+}\)). That this intermediate is observed in coated vesicles is expected on the basis of the 20% of the ATPase activity which was inhibited by strophanthidin. Finally, the absence of a detectable phosphorylated intermediate is consistent with the resistance of proton transport and Mg\(^{2+}\)-ATPase activity to inhibition by vanadate (10, 11), a potent inhibitor of only those cation transport ATPases which form a phosphorylated intermediate (36). The absence of a stable phosphorylated intermediate and resistance to vanadate are properties which the coated vesicle proton pump shares with the proton translocating ATPase of mitochondria (37, 38). However, proton pumping in coated vesicles has been shown not to be due to mitochondrial contamination by the resistance of proton transport and ATPase activity to oligomycin and aurovertin (10).

In was of interest to determine whether the coated vesicle proton pump carried out unidirectional transport of protons or whether countertransport of another cation occurred. Since Na\(^{+}\) and K\(^{+}\) are the most likely cations to be countertransported in vivo, we have measured \(^{22}\)Na\(^{+}\) and \(^{86}\)Rb\(^{+}\) (a K\(^{+}\) analog) movement during ATP-dependent proton uptake by coated vesicles (Table II). The transport studies were carried out in the presence of strophanthin and vanadate to prevent movement of \(^{22}\)Na\(^{+}\) and \(^{86}\)Rb\(^{+}\) due to the (Na\(^{+},K\(^{-}\))-ATPase activity present. Addition of ATP caused a small decrease in the amount of \(^{86}\)Rb\(^{+}\) trapped, but this was abolished by the addition of the proton ionophore carbonyl cyanide p-trifluoromethoxyphenylhydrazone, suggesting that \(^{86}\)Rb\(^{+}\) was
**Fig. 4. Phosphorylation of stripped vesicles with $^{32}$P.** Stripped vesicles (100 μg) were incubated for 2 h in 20 mM imidazole HCl (pH 7.5), 0.8 mM EGTA (lane B) plus 200 μM strophantidin (lane A) or plus 60 mM NaCl (lane C). After this incubation, 5 mM MgCl$_2$ was added followed by 200 μM $^{32}$P$_2$ (5 Ci/mmol, neutralized with imidazole). The samples were incubated for 1 min at 23 °C and then quenched and washed with 5% trichloroacetic acid and subjected to gel electrophoresis and autoradiography as described under "Materials and Methods". Positions on the gel are as described in Fig. 3.

**Table II**

| Isotope | Conditions | Gradient (+ATP/-ATP) |
|---------|------------|---------------------|
| $^{22}$Na$^+$ | 200 μM strophantidin, 20 μM vanadate, 1 mM MgCl$_2$ | 1.12 (±0.03) |
| $^{68}$Rb$^+$ | 200 μM strophantidin, 20 μM vanadate, 1 mM MgCl$_2$ | 1.01 (±0.04) |
| $^{32}$P$_2$ | 200 μM strophantidin, 20 μM vanadate, 1 mM MgCl$_2$, 5 μg/ml FCCP | 0.98 (±0.02) |
| $^{14}$C]Methylamine | 200 μM strophantidin, 20 μM vanadate, 1 mM MgCl$_2$, 5 μg/ml FCCP | 4.83 (±0.25) |

**Fig. 5. Equilibration of coated vesicles with $^{22}$Na$^+$ and $^{68}$Rb$^+$.** Coated vesicles (2.0 mg of protein/ml) were incubated at 23 °C in the standard buffer containing $^{22}$Na$^+$ (●; 30 μCi/ml) or $^{68}$Rb$^+$ (□; 50 μCi/ml). At the indicated times, 50-μl aliquots were assayed for trapped isotope as described under "Materials and Methods". The final level of trapping reached (approximately 0.04%/mg of protein/ml) was the same for both $^{22}$Na$^+$ and $^{68}$Rb$^+$ and was very close to the equilibrium level of trapping of the more permeable species $^{[14]}$C methylamine (0.036%/mg of protein/ml (10)).

In summary, we have identified NBD-Cl as a potent and ATP-protectable inhibitor of the coated vesicle proton pump. This pump does not appear to form a stable phosphorylated intermediate during turnover and does not appear to catalyze countertransport of another cation during proton uptake. Purification and reconstitution of this enzyme should provide additional information concerning the structure of this important proton transport system.

**Acknowledgments**—We thank Bertram Wiedenmann and Daniel Branton for providing the procedure used in purifying the clathrin-coated vesicles, for their electron microscopic analyses, and for many helpful discussions. We also thank Guido Guidotti and Gilbert Chin for their careful reading of the manuscript.

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5. The magnitude of the gradient depends in part on the proportion of coated vesicles containing a proton pump. If the rate of turnover of the proton pump is comparable to that of other transport ATPases (i.e. the (Na$^+$,K$^+$)-ATPase), the specific activity of the enzyme gives 1–2 copies/vesicle. Assuming a random distribution, this implies that 14–37% of the vesicles would contain no cation pump, which would result in apparent cation gradients of 2.7- and 7.1-fold, respectively.
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