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Clathrin-coated vesicles contain an ATP-dependent proton pump

(acidification of coated vesicles/endocytosis/receptors)

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ABSTRACT **Clathrin-coated vesicles isolated from calf brain contain an ATP-dependent proton pump. Proton movement was monitored by measuring [^{14}C]methylamine distribution. Addition of Mg^{2+} and ATP to coated vesicles equilibrated with [^{14}C]methylamine resulted in the generation of a 4- to 5-fold concentration gradient, corresponding to a ΔpH of 0.6–0.7 units between the medium and the acidic inside of the coated vesicles. ATP-dependent [^{14}C]methylamine uptake was abolished by the proton ionophore carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and partially inhibited by the carboxyl reagent *N,N'*-dicyclohexylcarbodiimide but was unaffected by the Na^+,K^+-ATPase inhibitors strophanthidin (100 μM) and vanadate (10 μM) and the mitochondrial ATPase inhibitors oligomycin (10 μg/ml) and aurovertin (1 μg/ml). GTP, but not the nonhydrolyzable analog 5'-adenylyl imidodiphosphate, could support [^{14}C]methylamine uptake. Dissipation of the membrane potential with K^+ and valinomycin resulted in stimulation of [^{14}C]methylamine uptake, whereas both FCCP and valinomycin stimulated the strophanthidin-resistant ATPase activity. These results are consistent with the existence of an electrogenic, ATP-dependent proton pump in clathrin-coated vesicles. This proton pump may play a role in the acidification events that are essential in receptor-mediated endocytosis.**

Acidification of intracellular compartments may be mechanistically involved in processes that normally occur soon after adsorptive or receptor-mediated endocytosis. For example, the nucleocapsid of several enveloped animal viruses enters the cytoplasm of their host cells only after the virus is endocytosed in coated vesicles and appears in intracellular compartments where low pH initiates a fusion reaction that transfers the viral genome into the host cell cytoplasm (1–3). Similarly, many proteins and hormones enter the cell after binding to specific receptors which are concentrated and internalized in coated pits and coated vesicles (4, 5). Here again, the newly endocytosed materials are soon found in a low pH compartment (6, 7) and the acidification that occurs in this compartment appears to be required to separate the receptor, which is recycled, from its ligand, which is further metabolized or itself recycled (7–12). It also has been suggested that exposure to low pH is required for entry of diphtheria toxin into the cytoplasm (13).

Although it is clear that newly endocytosed material may be exposed to low pH very soon after being internalized, the nature of the compartment in which acidification first occurs and the nature of the proton pump or pumps are not known. Recent studies that used fluorescently labeled α_2-macroglobulin indicate that acidification may begin almost immediately after formation of the endocytic vesicle (6, 10). Here, we show that acidification can occur in coated vesicles and that these coated vesicles contain an ATP-dependent proton pump.

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MATERIALS AND METHODS

Clathrin-coated vesicles were prepared from calf brain as described (14), except that the final material was passed twice over a 2 × 80 cm Sephacryl S-1000 column equilibrated with 100 mM 2-(*N*-morpholino)ethanesulfonic acid (Mes), pH 6.5/0.5 mM $MgCl_2$/1 mM EGTA and eluted at ≈40 ml/hr (15). The vesicles were stored in this buffer with 0.02% sodium azide at 4°C and were used within 2 weeks of preparation. By electron microscopy, 90–95% of the vesicles in our preparation were coated (with virtually all of the clathrin-coated structures containing a membrane vesicle). In addition, the preparation was shown to contain a low level of the lysosomal marker acid phosphatase (EC 3.1.3.2) (≈2–6% of the activity of purified lysosomes) and a similarly low level of the endoplasmic reticulum marker glucose-6-phosphatase (EC 3.1.3.9) (the activity was the same as that observed in erythrocyte membranes).

Coated vesicles were prepared for measurement of [^{14}C]-methylamine uptake or ATPase activity by a 1:50 dilution into 60 mM KCl/10 mM NaCl/10 mM Hepes, pH 7.5/0.10 mM EGTA followed by centrifugation at 100,000 × *g* for 45 min and resuspension of the pellet in the same buffer to a concentration of 1–3 mg of protein per ml. All measurements were carried out in this buffer unless otherwise noted. [^{14}C]Methylamine uptake was measured as follows. Washed vesicles were equilibrated for 2 hr at room temperature in the presence of 20 μCi of [^{14}C]methylamine (43 mCi/mmol; 1 Ci = 3.7 × 10^{10} Bq) per ml and in the presence or absence of various agents. Carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), valinomycin, strophanthidin, *N,N'*-dicyclohexylcarbodiimide (DCCD), oligomycin, and aurovertin were all added as ethanol solutions and the results were compared with controls containing an equivalent (0.5%) amount of ethanol (this level of ethanol had a negligible effect on both [^{14}C]methylamine uptake and ATPase activity). [^{14}C]Methylamine was completely equilibrated after 2 hr at room temperature (data not shown) and strophanthidin has previously been shown to be sufficiently membrane permeable to be able to inhibit reconstituted Na^+,K^+-ATPase activity under comparable conditions (16). After equilibration, either 1 mM ATP (trisodium salt) and 2 mM $MgSO_4$ or 3 mM NaCl and 2 mM $MgSO_4$ were added and the vesicles were incubated for an additional 2–10 min at 23°C. The reaction was stopped by placing the sample on ice and the amount of [^{14}C]methylamine trapped was determined by passing a 50-μl aliquot of the mixture over a 10-ml Sephadex G-50 column run at 1.5 ml/min at 4°C. The vesicle peak, which eluted from the column within 3–4 min, was collected in 0.5-ml fractions and the radioactivity was counted in Aquasol with a Beckman scintillation counter (the total [^{14}C]methylamine was determined by

Abbreviations: Mes, 2-(*N*-morpholino)ethanesulfonic acid; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide.

counting the radioactivity in an aliquot of the original mixture).

ATPase activity was measured by a continuous spectrophotometric assay with a Kontron model 810 spectrophotometer as described (17). Vesicles incubated as described above (except without [^{14}C]methylamine) were diluted 1:50 into assay mixture containing 60 mM KCl, 10 mM NaCl, 10 mM Hepes (pH 7.5), 0.10 mM EGTA, 1 mM ATP, 2 mM $MgSO_4$, 1.5 mM phospho*enol*pyruvate, 0.25 mg of NADH per ml, 20 μg of pyruvate kinase per ml, and 20 μg of lactate dehydrogenase per ml and the absorbance change at 340 nm was measured at 23°C. Acid phosphatase and glucose 6-phosphatase were assayed as described (18, 19) and protein concentrations were measured by the method of Lowry *et al.* (20) in the presence of 1% sodium dodecyl sulfate.

[^{14}C]Methylamine was obtained as an ethanol solution from New England Nuclear and was dried under nitrogen before use. ATP (vanadate free), strophanthidin, valinomycin, oligomycin, and the enzymes used in the coupled ATPase assay (both as ammonium sulfate suspensions) were purchased from Sigma. FCCP, DCCD, and Sephacryl S-1000 were obtained from Dupont, Aldrich, and Pharmacia, respectively. Aurovertin was the kind gift of Scott Thacher (Harvard University).

RESULTS AND DISCUSSION

As can be seen in Fig. 1, addition of Mg^{2+} and ATP to purified clathrin-coated vesicles equilibrated with [^{14}C]methylamine resulted in uptake of this species to give a 4- to 5-fold gradient.

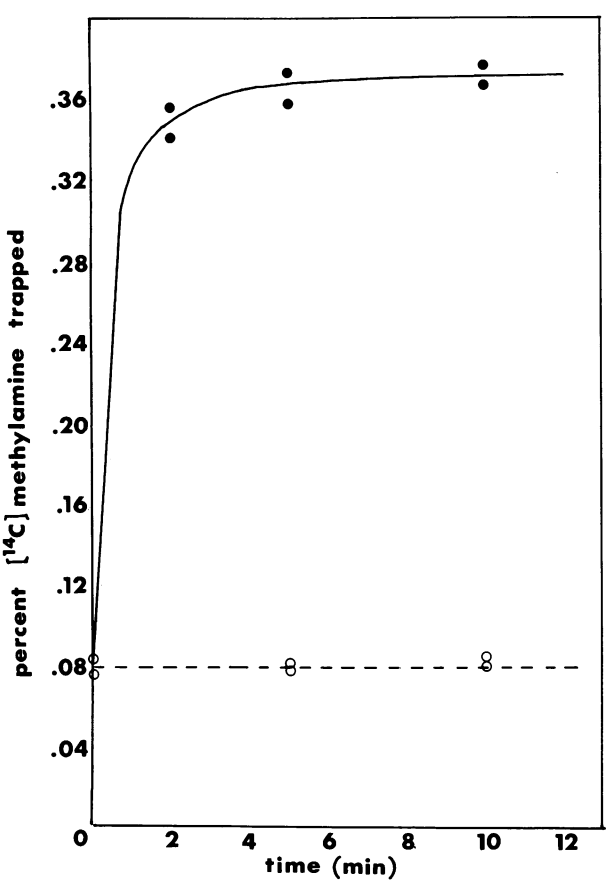

FIG. 1. ATP-dependent [^{14}C]methylamine uptake by clathrin-coated vesicles. Coated vesicles (2.2 mg of protein per ml) in 60 mM KCl/10 mM NaCl/10 mM Hepes, pH 7.5/0.1 mM EGTA were equilibrated with 20 μCi of [^{14}C]methylamine per ml for 2 hr at room temperature. At time zero, either 1 mM ATP (trisodium salt) and 2 mM $MgSO_4$ (●) or 3 mM NaCl and 2 mM $MgSO_4$ (○) were added, the vesicles were incubated for the indicated times at 23°C, the reaction then was stopped, and the amount of [^{14}C]methylamine trapped was determined.

Because [^{14}C]methylamine has been shown to accumulate in regions of low pH (21), the results imply that coated vesicles contain an ATP-dependent proton pump capable of generating a ΔpH across the vesicle membrane of 0.6–0.7 unit (inside acidic). This value is calculated by assuming equilibration of the unprotonated form of methylamine across the vesicle membrane and a pK_a of 10.5. Although some leakage of [^{14}C]methylamine may occur on separation of the vesicles at 4°C, this should not seriously alter the calculated ΔpH because this is dependent on the ratio of trapping observed in the presence and absence of ATP and [^{14}C]methylamine would be expected to have a similar half-time of leakage under either condition.

That the observed ATP-dependent uptake of [^{14}C]methylamine is due to coated vesicles rather than to the small amount of contaminating material is supported by two observations. First, nearly identical levels of ATP-dependent [^{14}C]methylamine uptake were observed for preparations containing 4% or 10% uncoated material as determined by electron microscopy [ATP-dependent gradients (mean ± average deviation) of 4.59 ± 0.09 and 4.47 ± 0.45 were observed for these preparations, respectively]. Second, when the uncoated material that was separated from coated vesicles on the first Sephacryl S-1000 column (15) was tested, virtually no ATP-stimulated [^{14}C]methylamine uptake occurred [a gradient (mean ± average deviation) of 1.02 ± 0.07 was observed]. Furthermore, when purified lysosomes are allowed to accumulate [^{14}C]methylamine under conditions comparable to those we used to assay acidification of coated vesicles, no more than a 10-fold accumulation of [^{14}C]methylamine occurs (21). To account for the observed 5-fold increase in [^{14}C]methylamine trapping on addition of ATP to our preparation, it would have to contain ≈50% lysosomes. This value exceeds by at least 10-fold the fraction of uncoated material observed in electron micrographs of our material and by 15-fold the level of lysosomal marker enzyme detected in biochemical assays (see *Materials and Methods*). That [^{14}C]methylamine uptake is not due to lysosomal contamination is supported further by the observation that although the uncoated material from the Sephacryl S-1000 column possesses twice the level of acid phosphatase activity as the purified coated vesicles, this uncoated material shows no ATP-dependent [^{14}C]methylamine uptake under our conditions.

In addition to ATP-stimulated [^{14}C]methylamine uptake, a Mg^{2+}-ATPase activity (mean ± average deviation) of ≈0.014 ± 0.003 μmol of ATP per min per mg of protein at 23°C was associated with our coated vesicle preparations. Although a number of laboratories have observed ATPase activity associated with coated vesicle preparations (22, 23), Rubenstein *et al.* (24) recently reported separating a "powerful" ATPase from their coated vesicles by agarose gel electrophoresis. Because specific activities of this ATPase were not reported and the detection limits of their assay system were not given, it is not clear whether Rubenstein *et al.* would have detected the level of ATPase activity reported in the present study. Moreover, it is possible that the conditions they employed (i.e., the absence of Na^+) precluded the observation of ATPase activity associated with their agarose-purified coated vesicles.

Table 1 shows the effects of various agents on ATP-dependent [^{14}C]methylamine uptake and ATPase activity of coated vesicles. That the observed [^{14}C]methylamine uptake represents a primary ATP-driven proton transport is supported by the observation that the proton ionophore FCCP both abolishes uptake and activates ATPase activity. In addition, the activating effect of valinomycin (in the presence of K^+) on both [^{14}C]-methylamine uptake and ATPase activity suggests that proton movement is electrogenic. Treatment of coated vesicles prepared in 60 mM KCl/20 mM Mes, pH 6.5/0.5 mM $MgCl_2$/1

Table 1. Effect of various agents on ATP-dependent [^{14}C]methylamine uptake and ATPase activity of coated vesicles

Incubation conditions	Nucleotide added	ATP-dependent gradient of [^{14}C]methylamine*	Relative strophanthidin-resistant ATPase activity†
Standard	ATP	4.59 (±0.09)	1.00
+5 μg of FCCP per ml	ATP	0.99 (±0.02)	1.50 (±0.16)
+3.3 μM valinomycin	ATP	5.69 (±0.01)	1.42 (±0.08)
+100 μM strophanthidin	ATP	4.73 (±0.05)	—
+10 μM vanadate	ATP	4.56 (±0.16)	0.98 (±0.04)
+150 μM DCCD	ATP	1.98 (±0.27)	0.60 (±0.10)
+10 μg of oligomycin per ml	ATP	4.95 (±0.05)	0.98 (±0.05)
+1 μg of aurovertin per ml	ATP	4.69 (±0.10)	1.00 (±0.04)
Standard	5′-Adenylyl imidodiphosphate	0.97 (±0.02)	ND
Standard	GTP	3.23 (±0.08)	ND
Na^+-free (70 mM KCl)	ATP	2.48 (±0.20)	0.59 (±0.08)
K^+-free (70 mM NaCl)	ATP	3.76 (±0.12)	ND

Coated vesicles (2.2 mg of protein per ml) were equilibrated under standard conditions (60 mM KCl/10 mM NaCl/10 mM Hepes, pH 7.5/0.1 mM EGTA) or with the salt replacements or additions indicated in the presence or absence of 20 μCi of [^{14}C]methylamine per ml for 2 hr at room temperature. At the end of this period, stimulation of [^{14}C]methylamine uptake by 1 mM of the nucleotide listed with 2 mM $MgSO_4$ was measured as described in the legend to Fig. 1 or the ATPase activity was measured as described in *Materials and Methods*. All values represent the average of at least two determinations, with the number in parenthesis being the average deviation from the mean. ND, not determined.

* Values represent the ratio of [^{14}C]methylamine trapped in the presence of nucleotide to that trapped in its absence after incubation for 5 min at 23°C.

† Values represent the relative strophanthidin-resistant ATPase activity under the conditions shown (except in the case of DCCD, the same concentration of the agent added prior to incubation was included in the assay mixture). The strophanthidin-resistant ATPase activity (which was 70–80% of the total) had a specific activity of ≈0.014 μmol of ATP per min per mg of protein at 23°C.

mM EGTA with valinomycin led to a larger ATP stimulation of [^{14}C]methylamine uptake (a gradient of up to 8-fold was observed), suggesting that in the case described in Table 1 insufficient K^+ may have leaked into the vesicles during the 2-hr incubation at room temperature to adequately dissipate any interior positive membrane potential which might be generated during proton uptake.

Because 20–30% of the ATPase activity associated with the coated vesicles was inhibited by strophanthidin (a specific inhibitor of the Na^+,K^+-ATPase), one possible mechanism of ATP-dependent proton uptake would involve generation of a Na^+ gradient (high intravesicular Na^+) by the Na^+,K^+-ATPase, followed by coupling of proton uptake to this Na^+ gradient via a Na^+/H^+ exchange system. Such coupled exchange systems have been demonstrated in a number of cell types (25). However, this possibility is excluded by the observation that ATP-dependent [^{14}C]methylamine uptake by coated vesicles is insensitive to both 100 μM strophanthidin and 10 μM vanadate (concentrations which completely inhibit Na^+,K^+-ATPase activity). Because vanadate appears to inhibit best those ATP-dependent ion pumps that involve a phosphorylated intermediate (26), this result also suggests that a covalent phosphorylated intermediate may not be involved in the mechanism of the coated-vesicle proton pump.

Both ATPase activity and ATP-dependent [^{14}C]methylamine uptake were partially inhibited by treatment with the carboxyl reagent DCCD [which inhibits most ATP-dependent ion pumps (27, 28)], but they were unaffected by the mitochondrial ATPase inhibitors oligomycin and aurovertin (27), indicating that uptake was not due to contaminating inverted mitochondrial particles. [^{14}C]Methylamine uptake was partially supported by GTP but was not supported by the nonhydrolyzable analog 5′-adenylyl imidodiphosphate. ATP-dependent uptake did not appear to specifically require the presence of either external Na^+ or K^+, but because the internal ion composition of the coated vesicles is not known, the data do not indicate anything about the internal ionic requirements of proton pumping.

CONCLUSIONS

Our results show the existence of an ATP-dependent proton pump in coated vesicles. Because many of the coated vesicles in our preparation must be derived, either *in vivo* or during the isolation procedure, from coated regions of plasma membranes, our results suggest that the ATP-dependent proton pump must exist in the cell membrane where it may be concentrated, together with certain receptors, in coated pit regions. It will be of interest to determine whether this or similar ATP-dependent proton pumps are common to many intracellular membranes, including coated regions derived from or part of the Golgi and endoplasmic reticulum membranes. As is the case for all currently available preparations of purified coated vesicles, the calf brain material we have examined probably represents a mixture of coated vesicles that originate from plasma membranes and from other intracellular membranes.

Because exposure to low pH appears to be an essential step in receptor-mediated endocytosis (see Introduction), the existence of a proton pump in the coated vesicles that represent the first compartment of the endocytic cycle is not surprising. Its pathway through the cell, its relationship to other proton pumps, such as those in lysosomes (21), its structural nature in comparison to other eukaryotic cation translocating ATPases (29), and the mechanism of its regulation must be the focus of further study.

Note Added in Proof. We have recently learned that Mellman and collaborators (C. J. Galloway, G. E. Dean, M. Marsh, G. Rudnick, and I. Mellman, personal communication) have observed ATP-dependent

acidification of endocytic vesicles isolated from cultured macrophages or fibroblasts. They used a lipid-impermeant pH-sensitive fluorescent probe to measure pH and their observations are in agreement with ours: ATP-dependent acidification can occur in endocytic vesicles very soon after their formation.

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