

Golgi Membranes Contain an Electrogenic H⁺ Pump in Parallel to a Chloride Conductance

JONATHAN GLICKMAN, KENNETH CROEN, SEAN KELLY, and QAIS AL-AWQATI

Departments of Medicine and Physiology, College of Physicians and Surgeons of Columbia University, New York, New York 10032

ABSTRACT Rat liver Golgi vesicles were isolated by differential and density gradient centrifugation. A fraction enriched in galactosyl transferase and depleted in plasma membrane, mitochondrial, endoplasmic reticulum, and lysosomal markers was found to contain an ATP-dependent H⁺ pump.

This proton pump was not inhibited by oligomycin but was sensitive to *N*-ethyl maleimide, which distinguishes it from the F₀-F₁ ATPase of mitochondria. GTP did not induce transport, unlike the lysosomal H⁺ pump. The pump was not dependent on the presence of potassium nor was it inhibited by vanadate, two of the characteristics of the gastric H⁺ ATPase. Addition of ATP generated a membrane potential that drove chloride uptake into the vesicles, suggesting that Golgi membranes contain a chloride conductance in parallel to an electrogenic proton pump.

These results demonstrate that Golgi vesicles can form a pH difference and a membrane potential through the action of an electrogenic proton translocating ATPase.

Many intracellular organelles are membrane bounded, a characteristic that allows the development of a microenvironment difference in composition is usually achieved by ion transport processes that are energized by cellular metabolism. In a number of cells and organelles including prokaryotes, mitochondria, lysosomes, and endocytic vesicles, proton electrochemical gradients generated by H⁺ pumps play an important role in signal transduction and energy conversion. We were stimulated by the recent discovery that monensin, an ionophore that exchanges H⁺ for sodium (or potassium), had dramatic effects on the structure and function of the Golgi apparatus (1, 2). Since the addition of monensin collapses a pH gradient, which could be the result of a H⁺ pump, we isolated Golgi vesicles and tested them for the presence of a proton pump. We found that a fraction of rat liver membranes, highly enriched in a Golgi marker, contained an electrogenic ATP-dependent proton pump. These membranes also appeared to contain a chloride conductance in parallel with this pump.

MATERIALS AND METHODS

Golgi membranes were prepared according to the procedure of Wibo et al. (3). Livers of starved rats were excised, weighed, finely minced with a razor blade, and then homogenized in 0.5 M sucrose with 20 strokes in a Dounce-type homogenizer fitted with a loose pestle in 2–3 ml of homogenizing buffer per gram tissue. Homogenizing buffer was 0.5 M sucrose which contained (as did all other sucrose solutions) 37.5 mM Tris-maleate, pH 6.5, 1% Dextran T-500 (Pharmacia, Inc., Piscataway, NJ), 5 mM MgCl₂, and 1 mM dithiothreitol, unless otherwise specified. The homogenate was centrifuged for 10 min at 2,100 rpm in a Sorvall centrifuge with the SA-600 rotor (DuPont Co., Instruments Products, Newtown, CT). The supernatant was decanted and the pellet

was resuspended in homogenizing buffer, homogenized with six strokes in a Dounce-type homogenizer (loose pestle), and centrifuged for 10 min at 1,800 rpm in a SA-600 rotor. The pooled supernatants were centrifuged for 7.9 min at 20,000 rpm in a Beckman ultracentrifuge with the SW-27 rotor (Beckman Instruments, Inc., Fullerton, CA), and the pellet was resuspended in homogenizing buffer and subjected to another centrifugation under the same conditions. The resulting pellet (microsomal fraction) was suspended in 37.4% sucrose to a final volume of 1 ml/g liver tissue, and a discontinuous sucrose density gradient was made in a 36-ml cellulose nitrate centrifuge tube as follows: 6.4 ml of 50.4% sucrose, 16.1-ml sample in 37.4% sucrose, 7.7 ml of 30.4% sucrose, 5.8 ml of 16% sucrose. The gradient was spun for 2.5 h at 24,000 rpm in a Beckman ultracentrifuge with the SW-27 rotor. Golgi membranes that collected at the 16–30.4% interface were aspirated with a syringe and diluted in 0.1 M sucrose, and spun for 30 min at 15,000 rpm in a Sorvall centrifuge. The pellets were resuspended in 0.1 M sucrose and either were used immediately for assays or were frozen at –70°C in the appropriate buffer containing 1 mM dithiothreitol and 3 mM ATP. When thawed, they were diluted in 20 ml of the appropriate buffer and pelleted again before use.

Enzyme Assays: ATPase activity was determined as previously described (4) and glucose-6-phosphatase activity was determined using the method of Baginski et al. (5). Galactosyl transferase was measured using ovalbumin as acceptor (6). *N*-acetyl-glucosaminidase activity was measured according to the method of Ray (7). Protein was measured using the Bio-Rad reagent (Bio-Rad Laboratories, Richmond, CA) with BSA as standard. All results are mean ± standard deviation.

Transport Assays: Fractions were isolated as described above and diluted in a buffer containing 150 mM KCl, 6 mM MgCl₂, 2 mM Tris, 2 mM (2, morpholino ethane sulfonic acid) MES, and 1 mM dithiothreitol, pH 7.0 and centrifuged at 15,000 rpm in a Sorvall centrifuge using an SM 24 rotor. (In some experiments sodium was substituted for potassium, or gluconate salts were used instead of chloride.) Aliquots of the pellets were suspended in 1.5 ml of the appropriate buffer containing 6 μM acridine orange, 50–70 μg/ml oligomycin, and 0.7 μM valinomycin unless otherwise specified. The sample was placed in a cuvette, and transport was initiated by adding ATP to a final concentration of 0.4 mM from a stock solution of 30 mM ATP that had been

titrated to pH 7.0 with 1 M Tris. The difference in absorbance at 492 and 540 nm was measured in a dual wavelength spectrophotometer (Johnson Research Foundation Instrument Shop, University of Pennsylvania, Philadelphia, PA). Nigericin was added to a final concentration of 1.3 μ M to collapse the pH gradient developed. For the membrane potential measurement, 1 μ M diS_C(5) was added to the vesicles, and the difference in absorbance was measured at 650 and 670 nm. Where inhibitors were used, samples were incubated with the specified inhibitor for 1 h on ice. When sulfhydryl reagents were used, dithiothreitol was omitted from the medium.

For measurement of chloride uptake, Golgi vesicles were initially frozen overnight in media containing 150 mM potassium or sodium gluconate, 6 mM Mg gluconate, 2 mM Tris, 2 mM MES, 1 mM dithiothreitol, and 3 mM ATP titrated to pH 7.0. When thawed, they were diluted in media of the same composition except for the absence of ATP. They were then pelleted at 20,000 rpm in a Sorvall centrifuge and resuspended in the same media using a 22 gauge needle and syringe to a final concentration of 1–1.5 mg/ml. The vesicles were then diluted into potassium gluconate transport media containing H³⁶Cl to a final concentration of 12 mM (specific activity 0.6 mCi/mmol). Valinomycin was added to a concentration of 1 μ M and ATP to a final concentration of 1.5 mM as indicated. The vesicles were incubated for 2 min and then filtered through Millipore filters (HAWP-025-00, 0.45 μ m; Millipore Corp., Bedford, MA) attached to a vacuum pump. The incubation test tube was rinsed with 2 ml of unlabeled medium at 4°C which was then used to wash the filter. An additional wash of 2 ml was finally included. The filters were then counted in a liquid scintillation counter.

RESULTS AND DISCUSSION

The method devised by Wibo et al. (3) gave a membrane fraction highly enriched in Golgi markers and quite depleted in markers of other organelles. As shown in Table I, this fraction contained 0.2% of the plasma membrane marker, the Na, K ATPase; 0.2% of the mitochondrial marker, the oligomycin-sensitive ATPase; 0.1% of the endoplasmic reticulum marker, glucose 6-phosphatase, and 0.4% of the lysosomal marker, *N*-acetyl-glucosaminidase. It was enriched in the Golgi enzyme, galactosyl transferase, containing 29% of the total content with a specific activity that was 129-fold higher than that of the initial supernatant (Table I). Hence, this fraction could reasonably be used for studies on the Golgi apparatus.

To test for ATP-dependent H⁺ transport we measured the uptake of acridine orange into these vesicles using a dual wavelength spectrophotometer (4). Acridine orange is a freely permeable weak base whose protonated form is much less diffusible. The development of an acid pH inside the vesicle traps the protonated form which will accumulate to a concentration proportional to the pH difference. At a high concen-

tration this planar dye forms dimers and higher order multimers with consequent spectral shifts. The dual wavelength spectrophotometer was tuned to the absorption characteristics of the monomer; hence a decline in the signal indicates uptake of the dye. As shown in Fig. 1, ATP induced a rapid uptake of acridine orange. To show that this uptake was due to a development of a pH gradient rather than to binding, we added nigericin, a proton ionophore that rapidly collapses a pH gradient by exchanging protons for potassium or sodium. Other protonophores such as gramicidin and carbonyl cyanide *m*-chlorophenyl hydrazone also rapidly collapsed the acridine orange signal (data not shown). As shown in the figure, nigericin "instantaneously" discharged the acridine orange, indicating that its accumulation was due to the development of a pH gradient. The rapid discharge also indicates that the acridine base is freely permeable and hence shows that the slower uptake induced by ATP is due to the slower development of a pH gradient rather than to the slow permeation of the dye. With this in mind, the initial rate of decline of the acridine orange signal could be used as some function of the initial rate of the proton pump.

Although Fig. 1 shows that the Golgi fraction contains an ATP-driven proton pump it is important to demonstrate that it is not due to contamination by inside-out mitochondrial membranes or by lysosomal fragments. We prepared rat liver submitochondrial particles by the method of Beyer (8) and found, as have others, that the mitochondrial H⁺ pump is exquisitely sensitive to oligomycin: as little as 25 ng/ml inhibits it completely (Fig. 2). All experiments reported here on Golgi vesicles were performed in the presence of 50 μ g/ml of oligomycin. Further, we found that the sulfhydryl reagent, *N*-ethyl maleimide, is a potent inhibitor of the Golgi pump (Fig. 3) whereas this agent had no effect on the mitochondrial ATPase (Fig. 2). Both, however, were inhibited by 200 μ M dicyclohexyl carbodiimide (not shown). The reagent NBD-Cl (7-chloro-4-nitrobenz-2-oxa-1,3 diazole) which reacts with sulfhydryl groups as well as with amino groups inhibited proton transport in the presence of dithiothreitol (Fig. 3) whereas its inhibitory effect on the chloroplast CF₁-ATPase and the mitochondrial F₁-ATPase is neutralized by dithiothreitol. These results clearly show that the Golgi proton pump is quite distinct from the mitochondrial F₁-F₀ H⁺ ATPase.

TABLE I
Subcellular Fractionation of Rat Liver Membranes

	Homogenate	Microsomes	Golgi
Total activities			
Na, K, ATPase	633 ± 51	166 ± 61	1.2 ± 0.6
Oligomycin sensitive ATPase	613 ± 150	534 ± 140	1.2 ± 1.0
Glucose 6-phosphatase	1,358 ± 488	772 ± 371	1.2 ± 0.6
Galactosyl transferase	5.6 ± 4.1	3.32 ± 1.57	1.65 ± 0.82
<i>N</i> -acetyl glucosaminidase	9.92 ± 0.12	1.26 ± 0.34	0.04 ± 0.03
Specific activities			
Na, K ATPase	4.28 ± 2.1	1.61 ± 0.97	6.67 ± 2.1
Oligomycin-sensitive ATPase	4.49 ± 0.47	8.51 ± 1.3	3.52 ± 1.33
Glucose 6-phosphatase	3.85 ± 0.8	8.16 ± 3.2	5.98 ± 3.4
Galactosyl transferase	3.59 ± 1.36	54.1 ± 13.1	464 ± 188
<i>N</i> -acetyl glucosaminidase	1.78 ± 0.42	9.76 ± 2.39	4.78 ± 1.9

Total enzyme activities are given in micromoles substrate hydrolyzed (or transferred) per hour. Specific activities are given in micromoles per milligram protein hour. Results were obtained from 3–7 preparations. In two additional preparations, the Na, K ATPase was assayed as strophanthidin-sensitive ATPase and compared with the simultaneously measured ouabain-sensitive ATPase. We found that the former assay gave twice the activity as the latter in the homogenate and microsomal fraction. All assays were done at 37°C.

It is now well established that lysosomes acidify their interiors by an ATP-driven proton pump. This ATPase is insensitive to oligomycin but is inhibited by sulfhydryl reagents such as maleimides (9). It is unlikely that the H^+ pump we describe here is due to a lysosomal contaminant since the Golgi fraction was quite depleted in the lysosomal marker, *N*-acetyl glucosaminidase. More convincingly, however, is the observation that rat liver lysosomes transport protons equally well in response to guanosine triphosphate as to ATP (10). Fig. 1 shows that the Golgi pump does not respond to GTP with H^+ pumping. These two results form strong evidence against the Golgi proton pump's being due to lysosomal contamination. This, however, should not be taken to mean that we believe that the lysosomal and Golgi pumps are fundamentally different enzymes. The effect of GTP in lysosomes may be due to the presence of an enzyme in the lysosomal membrane that can convert GTP to ATP.

A proton-translocating ATPase has recently been described in stomach which exchanges K^+ for H^+ and which is phos-

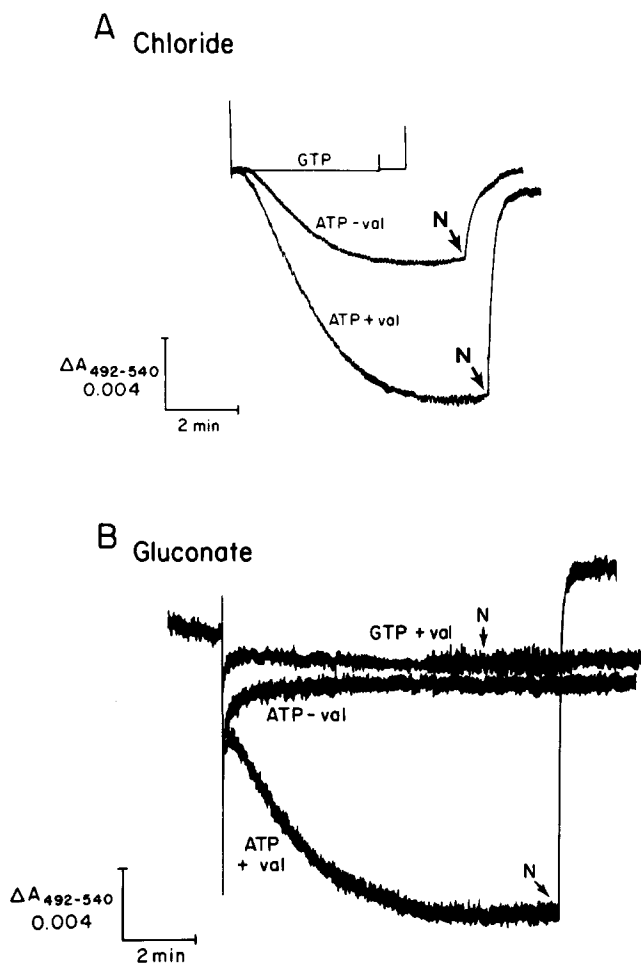


FIGURE 1 H^+ transport in Golgi vesicles measured as the uptake of acridine orange in a dual wavelength spectrophotometer as described in the text. Transport was initiated by the addition of ATP or GTP as labeled to a final concentration of 0.4 mM. Oligomycin was present in all assays in a final concentration of 50 μ g/ml. Valinomycin (Val), when present, was in a final concentration of 1 μ M. Nigericin was added at the end (labeled N) to a final concentration of 1.5 μ M. Each assay contained 150 μ g of protein. In A, the media were composed of chloride salts whereas in B they were composed of gluconate salts. The noise in the tracing is largely a function of the stirring rate.

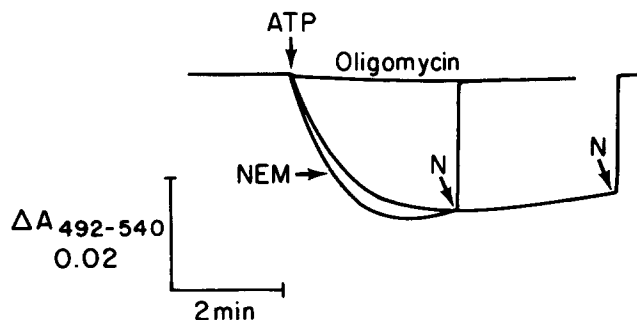


FIGURE 2 H^+ transport in submitochondrial particles. Assay conditions as described in the text and the legend to Fig. 1, except that no oligomycin was added except as shown where it was present in a concentration of 0.25 μ g/ml. *N*-ethyl maleimide (NEM) was present in a final concentration of 70 μ M. Valinomycin was present in all assays to a final concentration of 1 μ M. Nigericin was added to a final concentration of 1.5 μ M at N. Each assay contained 50 μ g of protein.

phorylated during its catalytic cycle (11). O'Neal et al. (12) has shown that vanadate is an agent that inhibits Ca ATPase as well as Na, K ATPase but not the mitochondrial F_0F_1 ATPase, and suggested that this reagent might inhibit only phosphorylated ATPases. In agreement with this suggestion is the observation that the gastric H^+K^+ ATPase is inhibited by vanadate (11). We think that the Golgi ATPase is different from the gastric ATPase since it does not require K^+ , nor is it inhibited by vanadate. Golgi vesicles were loaded with either potassium or sodium media by freezing the membranes overnight in the buffers, followed by slow thawing and shearing the vesicles using a fine needle and syringe in the same media. The initial rate of H^+ transport in potassium-loaded vesicles diluted into potassium media was (in absorbance units $\times 10^{-3}/\text{min mg protein}$) 29.5 ± 1.9 , whereas that for sodium-loaded vesicles assayed in sodium media without potassium was 28 ± 3.0 . When these vesicles were preincubated and assayed in the presence of 50 μ M vanadate, the initial rate was 24.5. The lack of effect of vanadate suggests that this H^+ pump was not phosphorylated during its catalytic cycle. Further, unlike the neutral gastric pump, the golgi H^+ ATPase was "electrogenic" (see below).

When ATP hydrolysis was measured in the presence of oligomycin, ouabain, and EGTA with and without 100 μ M *N*-ethyl maleimide, we found an activity that was barely detectable in the homogenate. In the crude microsomes there was 0.68 μ mol/h mg protein which increased by a factor of three in the Golgi fraction. This "*N*-ethyl maleimide-sensitive" ATPase activity was completely inhibited by dicyclohexyl carbodiimide but only slightly inhibited by vanadate. Clearly, more work needs to be done before this ATPase activity can be identified as the enzymatic equivalent of the H^+ pump.

An important characteristic of any ion pump is whether it can generate a membrane potential. We tested for this electrogenic behavior using three independent methods. In the first, we measured H^+ transport in the presence and absence of valinomycin, a conductive carrier of K^+ that in the presence of K^+ on both sides of the membrane can collapse a membrane potential. If the H^+ pump was electrogenic, then a positive membrane potential will develop immediately after the addition of ATP. A fundamental characteristic of a tightly coupled electrogenic proton pump is that an unfavorable potential will slow down the rate of transport and the rate of

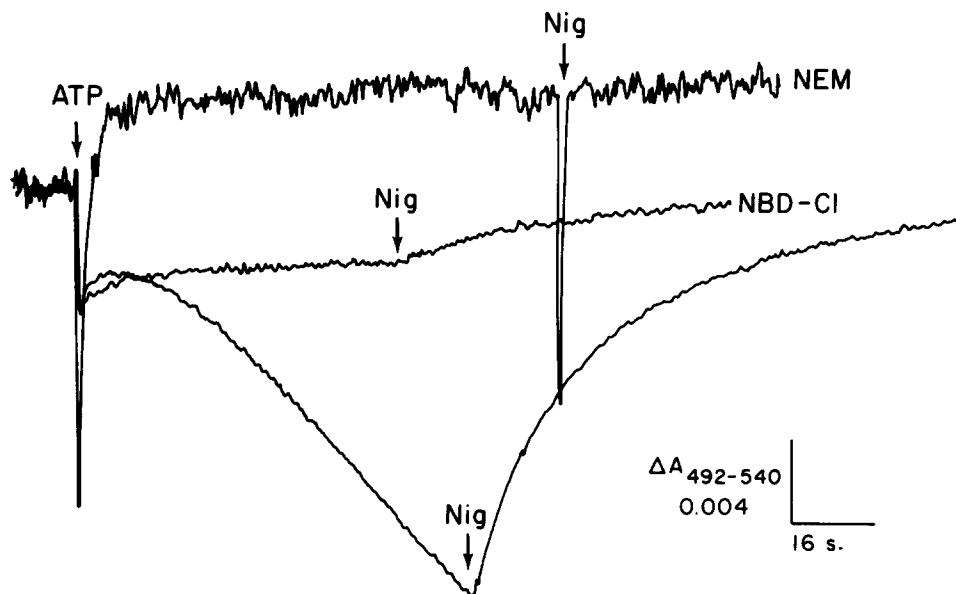


FIGURE 3 H^+ transport in Golgi vesicles. The effect of *N*-ethyl maleimide (*NEM*) (final concentration $20 \mu M$) and NBD-Cl, final concentration $100 \mu M$. Each assay contained $70 \mu g/ml$ oligomycin, $1 \mu M$ valinomycin, and $150 \mu g$ of vesicle protein. $1 mM$ dithiothreitol was also present in the control and NBD-Cl but not the *N*-ethyl maleimide experiments. Nigericin was added to a final concentration of $1.5 \mu M$ at *Nig*. Otherwise, it was as described in the text.

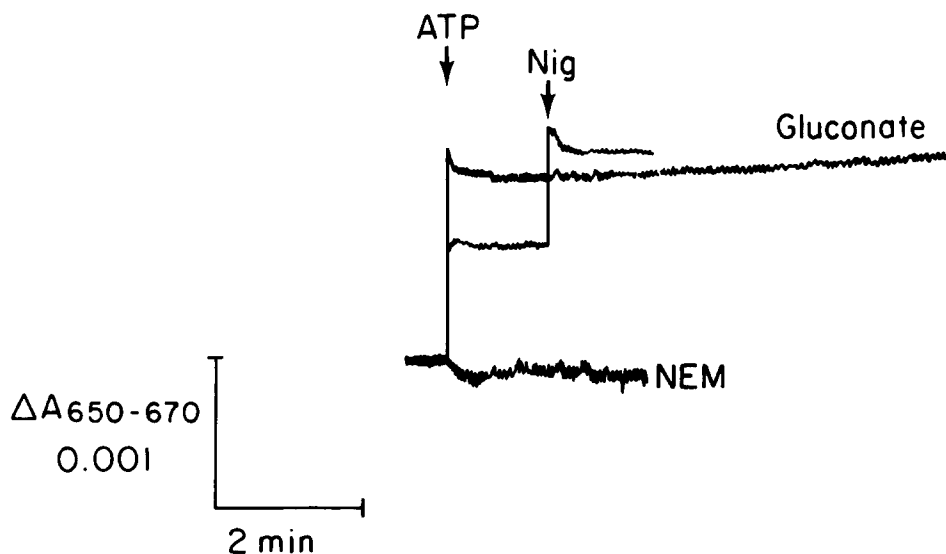


FIGURE 4 Measurement of membrane potential in Golgi vesicles using the ejection of the carbocyanine dye diS $C_3(5)$. Dye was present in $1 \mu M$ concentration. Transport was initiated by addition of $0.4 mM$ ATP. In one assay, *N*-ethyl maleimide was also present to a final concentration of $50 \mu M$ (tracing labeled *NEM*). In the other trace, Nigericin was added after ATP to a final concentration of $1.5 \mu M$ (trace labeled *Nig*). Both of these assays were performed in the presence of chloride as the anion. In another assay, vesicles were preincubated in potassium gluconate media (tracing labeled *gluconate*).

ATP hydrolysis (13, 14). Fig. 1*A* shows that addition of valinomycin accelerated the rate and increased the extent of development of a H^+ gradient in KCl media. When these experiments were repeated using potassium salts of the less permeant anion, gluconate, there was no development of pH gradients unless valinomycin was also included in the media (Fig. 1*B*). These results suggest that chloride was able to partially shunt the membrane potential generated by the proton pump, implying the existence of a chloride conductance in parallel with an electrogenic pump.

We also used the carbocyanine cationic dye DiS $C_3(5)$ to test for the development of a membrane potential (15). In the dual wavelength spectrophotometer tuned to the difference in absorbance between membrane-bound and free dye, we found that ATP induced an ejection of the cationic dye from the Golgi vesicles, compatible with the presence of an electrogenic pump causing a positive potential inside the vesicle (Fig. 4). Vesicles pretreated with *N*-ethyl maleimide showed no ejection of the cation, suggesting that the signal generated is coupled to the ATPase activity. Addition of nigericin, which is a neutral K:H exchanger, should collapse the pH gradient

(as shown in Figs. 1–3) but should increase the membrane potential, which is what we found (Fig. 4). This behavior is analogous to the previously demonstrated effect of valinomycin on the development of a pH gradient (Fig. 1). Addition of ATP to Golgi vesicles bathed with gluconate caused a signal (i.e., membrane potential positive inside) that was larger than that seen in the presence of chloride (Fig. 4), demonstrating the presence of a chloride conductance in parallel with the H^+ pump in the same vesicles. These results demonstrate one of the characteristics of a tightly coupled electrogenic proton pump where the rate of transport is affected by the net electrochemical gradient rather than by one of the components of that gradient (13, 14).

Unfortunately, addition of valinomycin, carbonyl cyanide *m*-chlorophenyl hydrazone, or gramicidin to generate or collapse a membrane potential produced a large artifactual signal that was probably due to interaction of these ionophores with the dye bound to these vesicles. Hence, we were unable to calibrate the signal against known imposed potentials. However, the fact that the signal induced by ATP increased on the addition of nigericin or substitution by gluconate and was

TABLE II
Potential Driven Uptake of ^{36}Cl into Golgi Vesicles

In	Out	Val	ATP	^{36}Cl uptake nmol/mg · 2 min
K diffusion potentials				
Na	K	+	~	$63.2 \pm 10^*$
Na	K	-	-	40.8 ± 4.3
K	K	-	-	44.4 ± 6.6
K	K	+	-	38.5 ± 6.1
ATP-induced Uptake				
K	K	-	+	$34.7 \pm 5.9^*$
K	K	+	+	23.9 ± 6.0

ATP was added in 1.5-mM and valinomycin in 1- μM concentrations. Results are given as mean \pm SD of six experiments.
* $p < 0.001$ from appropriate control.

prevented by pretreatment with *N*-ethyl maleimide supports the contention that it is due to a pump-generated potential rather than an ATP-induced artifact.

To provide more direct evidence for the electrogenicity of the pump and for the presence of a parallel chloride conductance, we measured the uptake of radioactive chloride into these vesicles. Golgi membranes were loaded with either potassium or sodium gluconate and diluted into potassium media containing ^{36}Cl with or without valinomycin to generate a potassium diffusion potential. Table II shows that these vesicles accumulated chloride only when an inwardly directed potassium gradient was imposed in the presence of valinomycin, indicating that these membranes possess a chloride conductance. (The lack of effect of inwardly directed potassium gradients in the absence of valinomycin indicates that these membranes either do not have a potassium conductance or cannot distinguish between sodium and potassium.) Table II shows the results of another experiment where addition of ATP induced the uptake of ^{36}Cl in the absence of potassium gradients. Addition of valinomycin to collapse a pump-generated potential prevented the ATP-induced chloride uptake.

The three types of experiments presented in Figs. 1 and 4 and in Table II provide strong evidence that the Golgi H^+ pump is electrogenic and is located in membranes that possess a chloride conductance but little sodium/potassium selectivity. These results also demonstrate an important characteristic of an electrogenic proton pump in which the gradients generated by the pump could be varied. Collapsing the pH difference by a neutral proton carrier will increase the positive membrane potential (Fig. 4). Collapsing the membrane potential by an ion conductance will increase the pH difference (Fig. 1). This behavior could form the basis for an attractive new mechanism of regulation since cellular mechanisms may exist that could act as neutral proton carriers or as ion conductors. It is becoming apparent that the binding of some ligands to their receptors results in a change in ion conductance thought to be due to opening of a nonselective cation channel (in the case of Fc-receptor-Ig interaction) (16) or to the closure of a potassium channel in the case of the binding of thyrotropin-releasing hormone to its receptor in pituitary cells (17). A tightly coupled proton pump thus offers a unique opportunity for the cell to regulate two fundamental driving forces in small compartments by changing the conductances in parallel with the pump. Changes in the chloride conductance of these vesicles by some regulatory influence could

change the fundamental properties of these vesicles, converting them to acid vesicles with no membrane potential or neutral vesicles with a high potential and thereby possibly changing their behavior. These regulatory influences may also pose some risks for the unwary investigator. The absence of a pH gradient (the parameter usually measured) should not be interpreted as an absence of proton pumps or the presence of defective pumps since it could be due to closure of the chloride conductance and hence reduction of the pH difference due to the development of a large membrane potential.

This proton pump is remarkably similar to the H^+ translocating ATPase which we have recently described in the turtle bladder and kidney (4, 18). This renal H^+ pump is packaged in endocytic vesicles located underneath the apical plasma membrane. Stimuli to urinary acidification caused fusion of these vesicles with the apical membrane, thereby inserting these pumps into the membrane and increasing the rate of transepithelial transport (19). This endocytic proton pump is also similar in behavior to that described in chromaffin granules (20, 21), and we also have evidence which shows that it is also present in brain coated vesicles (A. Lowe and Q. Al-Awqati, unpublished observations) and in rough and smooth endoplasmic reticulum (R. Rees-Jones and Q. Al-Awqati, submitted for publication).

As this paper was being prepared for submission, reports appeared that showed the presence of a proton pump in brain coated vesicles and in fibroblast and macrophage endocytic vesicles (22-24). These pumps are similar to the one described here. Such studies raise the question of whether the H^+ pump we describe is not the result of contamination of the Golgi vesicles with coated vesicles (and, of course, vice versa). Since coated vesicles bud off and fuse with Golgi membranes, the most reasonable interpretation is that the proton pump is present in the two types of membranes. However, without immunocytochemical localization, this question cannot be resolved.

The role that the low pH and membrane potential plays in the function of the Golgi apparatus is not clear at present. It is likely that it may participate in some way with the fusion process in which these vesicles are especially active. Other possible functions include serving as a driving force for sugar transport into the Golgi apparatus and in sorting of secreted proteins.

There is increasing evidence that the Golgi apparatus is functionally as well as structurally heterogeneous. Localization of the proton pump to any one of the components of the Golgi apparatus must await more definitive immunocytochemical methods.

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