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Isolation of rat liver endocytic vesicles using the proton pump as a marker

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ATP-driven acidification visualized by the ΔpH indicator acridine orange was used as marker for isolation of endocytic vesicles from rat liver. By differential and Percoll density gradient centrifugation, a vesicle fraction was obtained with an approx. 80-fold enriched H^+-pump activity. The preparation contained vesicles that had taken up fluorescein isothiocyanate-labeled dextran or horseradish peroxidase injected into rats in vivo, proving the presence of endosomes. The H^+-pump in these vesicles showed: (a) strict preference for ATP; (b) stimulation by Mg^{2+} and Mn^{2+}, but not by monovalent cations; (c) stimulation by Cl^-, I^- and Br^-; (d) electrogenicity; (e) insensitivity to vanadate, slight inhibition by oligomycin and strong inhibition by *N*-ethylmaleimide (NEM) and *N,N'*-dicyclohexylcarbodimide (DCCD). The vesicles exhibited an ouabain-, oligomycin- and levamisole-resistant ATPase activity, which was slightly stimulated by Cl^-, unaffected by vanadate and inhibited by NEM and DCCD. Thus, a simple and efficient high-speed centrifugation method is available for isolation of endocytic vesicles from mammalian liver.

Introduction

Endosomes are intracellular organelles that belong to the endo- and exocytotic pathway, termed collectively as the vacuolar system. This system serves for uptake of nutrients and other extracellular material, internalisation of receptor-bound molecules, such as growth factors, hormones, antibodies, and lipoproteins, and recycling of the plasma membrane components [1,2]. Because of their heterogeneous morphology and the absence of a marker enzyme, the isolation of these vesicles is difficult. The only transport mechanism known to exist in endosomes is an ATP-dependent, anion-stimulated proton-translocating ATPase (H^+-pump), which is of great importance for the physiology of these vesicles [3]. However, as an H^+-pump with similar characteristics is present also in all other components of the vacuolar system [3], no attempts have been made to use this pump as a marker in the isolation procedure of endosomes. Rather, a variety of alternative methods have been developed to isolate endocytic vesicles from various cells, in which the use of ultracentrifuges, special gradients or a free-flow electrophoresis system is required [4–11].

In a method recently developed by us [4,12], we successfully used the H^+-pump activity as a marker to separate renal cortical endosomes from other

Abbreviations: CCCP, carbonylcyanide *p*-chloromethoxyphenylhydrazone; DCCD, *N,N'*-dicyclohexylcarbodiimide; FITC-dextran, fluorescein isothiocyanate-labeled dextran; H^+-pump, proton-translocating ATPase (H^+-ATPase); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HRP, horseradish peroxidase; NEM, *N*-ethylmaleimide; Oxonol V, bis(3-phenyl-5-oxoisoxazol-4-yl)pentamethione oxonol.

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cellular membranes. The preparation involved differential centrifugation and a single Percoll density gradient, and required only a high-speed centrifuge. A similar approach was used in this work to isolate endocytic vesicles from rat liver homogenates. In isolated vesicles, we have characterized the H^+-pump.

Materials and Methods

Preparation of liver endocytic vesicles. Endocytic vesicles were isolated from rat liver homogenate by differential and Percoll density gradient centrifugation using a Sorvall RC5B centrifuge (rotor SS34). In a standard experiment, two rat livers were pooled and homogenized in 70 ml homogenizing buffer (300 mM mannitol/12 mM Hepes-Tris (pH 7.4)) in a motor-driven loose-fitting glass/Teflon Potter homogenizer with five strokes at 1200 rpm. The homogenate was diluted with an additional 70 ml of homogenizing buffer and the whole sample was centrifuged at $2500 \times g$ for 15 min. The pellet was discarded. The supernatant (S_1) was centrifuged at $20000 \times g$ for 20 min and the resulting pellet was discarded. The supernatant (S_2) was centrifuged at $48000 \times g$ for 30 min. The supernatant was carefully removed by sucking. The upper, very fluffy pellet (P_3) was saved, whereas the remaining, harder jelly pellet was discharded. P_3 was dispersed with 30 ml homogenizing buffer and homogenized in a tight-fitting glass/Teflon homogenizer. To 32 g of suspension, 6.1 g of concentrated Percoll was added (final concentration of Percoll, 16% w/w). After mixing, the sample was centrifuged at $48000 \times g$ for 30 min. The resulting self-oriented gradient was fractionated in 1.0 ml fractions. The first 5 ml from the top of the gradient were discarded. The next 7 ml (fractions 6–12), which exhibited the highest H^+-pump activity, were used for the further purification steps. The rest of the gradient was discarded. The fractions 6–12 were pooled (PG_2) and diluted with 30 ml potassium gluconate buffer (300 mM mannitol/100 mM potassium gluconate/5 mM $MgSO_4$/5 mM Hepes-Tris (pH 7.0) and kept in an ice-bath for 30 min. The suspension was then centrifuged at $48000 \times g$ for 30 min. The supernatant was sucked off. The pellet (P_4) was resuspended in about 1.5 ml potassium gluconate buffer and transferred into an Eppendorf cup. By using a cup adapter, the suspension was centrifuged at $2500 \times g$ for 15 min. The resulting supernatant (S_5) was carefully transferred using a pipette into another Eppendorf cup. The pellet was discarded. S_5 was centrifuged at $48000 \times g$ for 30 min. The resulting supernatant was sucked off. The pellet consisted of two layers; the upper white, fluffy one, which contained vesicles highly enriched in H^+-pump activity, and the lower, much bigger and reddish one, with much less H^+-pump activity. The upper fluffy pellet (endocytic vesicles) was carefully removed by using a tuberculine syringe with a needle and used for transport studies.

Loading of endocytic vesicles with FITC-dextran and horseradish peroxidase (HRP). FITC-dextran (40 mg in 0.5 ml Ringer's solution) was injected i.v. as a single dose into rats anesthetized with Inactin (150 mg/kg). 10 min later, the livers were perfused with a cold Ringer's solution under approx. 150 cm water pressure for 5 min, removed and used for isolation of endocytic vesicles.

For histochemical analyses, HRP (50 mg HRP in 0.5 ml Ringer's solution) was injected i.v. into anesthetized rats. After 15 min, the livers were removed and the animals were killed. Small pieces of the tissue and, later, isolated vesicles were fixed for 60 min with 2.5% glutaraldehyde in 0.1 M sodium cacodilate buffer (pH 7.3). The material was washed overnight in 0.05 M Tris-HCl buffer (pH 7.4) plus 7.5% sucrose and then incubated for 20 min in a buffer comprising 0.05% diaminobenzidine and 0.01% H_2O_2 as the substrate for peroxidase [13]. The peroxidase reaction was stopped by a washing step and by fixation with 1% OsO_4. The samples were block-stained with 2% uranyl acetate, dehydrated and embedded in an epoxy resin. Sections were viewed in a Philips 300 electron microscope.

Protein and enzyme assays. Protein was determined by the method of Bradford [14]. Leucine arylamidase (EC 3.4.11.2), acid phosphatase (EC 3.1.3.2), alkaline phosphatase (EC 3.1.3.1), and acetylcholinesterase activities (EC 3.1.1.7) were measured by using commercial kits (Merckotests No. 3359, 3305, 3344 and Monotest No. 124125, respectively). $(Na^+ + K^+)$-ATPase activity (EC 3.6.1.3) was determined by the coupled optical test

as described in Ref. 15. The activities of succinate–cytochrome-*c* oxidoreductase (EC 1.3.99.1), KCN-resistant NADH oxidoreductase (EC 1.6.99.2) and β-glucosaminidase (EC 3.5.1.33) were measured by the methods described in Refs. 16, 17 and 18, respectively. The activity of 5′-nucleotidase (EC 3.1.3.5) was determined at either pH 5.0 (acid 5′-nucleotidase) or pH 8.4 (alkaline 5′-nucleotidase), as described by Burnside and Schneider [19]. The method described by Novikoff and Heuss [20] was used to study thiamin pyrophosphatase activity (EC 3.6.1.6). Phosphate liberated in these reactions was determined colorimetrically [21]. H^+-ATPase activity in isolated vesicles was measured by the coupled optical assay, as described by us previously [12].

Measurement of H^+-pump activity. The H^+-pump activity was measured by acridine orange fluorescence quenching, as described in detail previously [4,12]. Acridine orange is a fluorescent indicator of ΔpH, but not of the membrane potential [22]. Before the measurements were made, isolated vesicles were adjusted to a protein concentration of 4–6 mg/ml by adding potassium gluconate buffer and preincubated at room temperature for 2 h. In a typical assay, an aliquot of isolated vesicles (protein amount indicated in the corresponding figures and tables) or liver homogenate (0.4–0.8 mg protein) was added into 2.0 ml of potassium gluconate or KCl buffer (300 mM mannitol/100 mM KCl/5 mM $MgSO_4$/5 mM Hepes-Tris (pH 7.0)) that contained 6 μM acridine orange and ionophores and inhibitors as required. Inhibitors and ionophores were added from water (NEM, vanadate) or ethanol stocks (oligomycin, CCCP, DCCD) in the final concentrations indicated in the figure legends. Controls contained 1% of ethanol. If not otherwise stated, the vesicles were preincubated with the indicated ionophores and inhibitors at room temperature for 15 min before starting the reaction. The activity of the H^+-pump was initiated by adding ATP from an aqueous stock solution (final concentration of ATP, 1.5 mM). The change in fluorescence was continuously recorded under constant stirring at room temperature (excitation, 493 nm; emission, 525 nm).

In vesicles with trapped FITC-dextran, the conditions for the measurement of the H^+-pump were the same as described above for the acridine orange method. The decrease of intravesicular FITC-dextran fluorescence was recorded following addition of 1.5 mM (final concentration) ATP (excitation, 492 nm; emission, 515 nm).

When necessary, the rate of acidification (ATP-driven H^+-pump activity) was estimated by drawing a tangent to the initial part of the fluorescence recording, and was expressed as fluorescence change per min (ΔF/min). The initial rates in the presence of ATP were corrected for the fluorescence changes recorded in the absence of ATP. All experiments were performed in a range in which fluorescence changes were proportional to protein concentration, i.e., using not more than 0.2 and 0.8 mg of vesicle and homogenate protein, respectively.

Determination of membrane potential. Changes in membrane potential were visualized by the fluorescent voltage-sensitive dye, Oxonol V, a compound used previously to test for electrogenicity of H^+-pump in rat liver clathrin-coated vesicles [23] and multivesicular bodies [24]. This negatively charged dye partitions between intra- and extravesicular fluid in response to membrane potential. At an inside-positive membrane potential, the dye is accumulated in the vesicles, causing a decrease in its fluorescence. Vesicles were added to 2 ml potassium gluconate or KCl buffer that contained 2.9 μM Oxonol V, and ionophores and inhibitors as required in concentrations indicated in the figure legends. Before adding ATP (final concentration, 1.5 mM), the vesicles were preincubated with ionophores or inhibitors (or ethanol in control experiments) at room temperature for 10 min. The fluorescence was continuously recorded under constant automatic stirring of the sample (excitation, 622 nm; emission, 669 nm).

The studies were made either with freshly isolated vesicles or with vesicles which had been kept in liquid nitrogen for 1–3 days. In a later case, before performing the measurements, the vesicles were thawed at 37°C. No qualitative differences in data were observed between the fresh and the thawed endosomes, however, after longer storage in a frozen state, for unknown reasons, the endosomes exhibited a weaker ATP-driven H^+-pumping ability.

The recordings are from single experiments and

are representative of three or more repetitions with separate membrane preparations. Other data are shown as means ±S.E of the number of experiments indicated in the legends.

Materials. Acridine orange was from Eastman Kodak (Rochester, NY, U.S.A.), FITC-dextran (M_r 17500) from Sigma (St. Louis, U.S.A.). oligomycin and NEM from Serva (Heidelberg, F.R.G.), DCCD from Calbiochem (Kalamazoo, MI, U.S.A.), Percoll from Pharmacia Fine Chemicals (Uppsala, Sweden), vanadate (Na_2HVO_4) from Merck (Darmstadt, F.R.G.), and Oxonol V from Molecular Probes, Inc. (Junction City, OR, U.S.A.). CCCP, HRP (250 U/mg), and ATP (disodium salt) were purchased from Boehringer (Mannheim, F.R.G.). Other chemicals were of analytical grade.

Results

Isolation of liver endocytic vesicles

Previously, it has been shown that endocytic vesicles from various cells contain an ATP-driven, anion-stimulated H^+-pump, but no other marker enzyme [3,4,6–9,11,12,25]. Thus, in order to isolate liver endosomes, the rats were injected with FITC-dextran, and the H^+-pump activity was traced throughout the purification steps by recording the ATP-dependent changes in fluorescence of either intra-vesicularly trapped FITC-dextran or of acridine orange. The membrane fraction with the highest ATP-dependent, Cl^--stimulated H^+-pump activity obtained by differential centrifugation was separated by a 16% w/w Percoll density gradient (Fig. 1). Each fraction of the gradient was tested for the FITC-dextran fluorescence (triangels) and the H^+-pump activity measured by either FITC-dextran (open circles) or acridine orange fluorescence changes (closed circles) in the presence of Cl^- and ATP. The FITC-dextran fluorescence distributed all over the gradient, showing two broad peaks; the lower peak was in fractions 6–14, and the higher one in fractions 25—33. The H^+-pump activity, measured by either FITC-dextran or acridine orange, comigrated with the lower peak in the top fractions of the gradient. The fractions with the highest H^+-pump activities measured with FITC-dextran (fractions 6–12, hatched area) were pooled and processed further by differential centrifugation.

As measured in final vesicle preparations with either in vivo trapped FITC-dextran or with externally added acridine orange (Fig. 2A and B), ATP

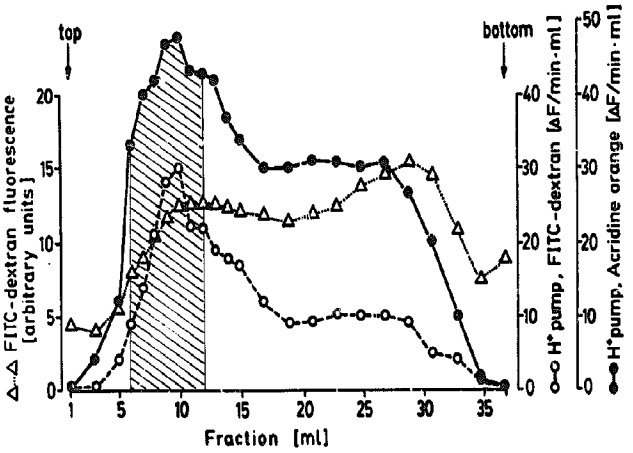

Fig. 1. Distribution of FITC-dextran fluorescence (Δ) and H^+-pump activity measured by acridine orange fluorescence quenching (●) or FITC-dextran fluorescence decrease (○) along a Percoll gradient. A 0.1 ml aliquot of each fraction was diluted into 1.9 ml KCl-buffer in the absence (Δ, ○) or presence of 6 μM (final concentration) acridine orange (●). H^+-pump activity was recorded by the initial rates of acridine orange fluorescence quenching or FITC-dextran fluorescence decrease following addition of 1.5 mM ATP (final concentration).

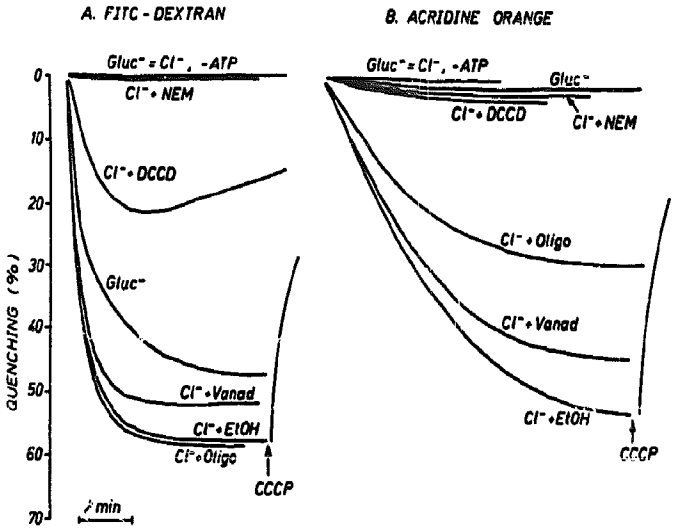

Fig. 2. Cl^--stimulated H^+-pump activity in absence (–ATP) or presence of ATP (all other experiments) in isolated liver endocytic vesicles as measured by fluorescence changes of incorporated FITC-dextran (A) or of acridine orange (B): effect of various inhibitors. Vesicles (0.068 mg protein in A and 0.123 mg protein in B), preloaded with gluconate buffer, were diluted into the same ($Gluc^-$) or KCl-buffer (Cl^-) that contained no (A) or 6 μM acridine orange (B) and the indicated inhibitors in the following (final) concentrations: oligomycin (Oligo), 10 μg/ml; vanadate (Vanad), 0.5 mM; DCCD, 0.5 mM; NEM, 0.5 mM. Controls contained 0.5% ethanol (EtOH). Before adding ATP to start the H^+-pump, the vesicles were incubated in the presence of ethanol or inhibitors at room temperature for 15 min. At the indicated time (arrow) the protonophore CCCP was added (final concentration 10 μM) to dissipate the pH gradient.

elicited a time-dependent intravesicular acidification. This acidification was more pronounced in the presence of chloride than with gluconate, indicating the presence of an ATP-driven, anion-stimulated H^+-pump in the vesicles. The Cl^- stimulated H^+-pump measured by FITC-dextran (Fig. 2A) was not inhibited by oligomycin or vanadate. DCCD inhibited strongly and NEM completely. In acridine orange studies (Fig. 2B), vanadate was not inhibitory, oligomycin inhibited partially, and DCCD and NEM inhibited completely. Although some differences exist in two assays with respect to stimulation of the H^+-pump activity with gluconate and inhibition by oligomycin, possibly due to a different sensitivity of fluorescent dyes to ΔpH (acridine orange has a low sensitivity to ΔpH below 0.5 pH units [22]), the overall inhibitory pattern in both assays is comparable and remarkably similar to the pattern previously established for the H^+-pump in rat renal endocytic vesicles [4,12]. Furthermore, as in renal endosomes, the proton gradient established in liver endocytic vesicles was not affected by addition of Na^+, indicating the absence of an Na^+–H^+ exchanger in these membranes (data not shown).

The experiments with FITC-dextran clearly showed that our preparations of liver vesicles contained endocytic vesicles that have incorporated this fluorescent fluid phase marker in vivo. To further visualize directly the endosomes in our vesicle preparation, the rats were injected with HRP, an enzyme that has been shown to be taken up by liver endocytic vesicles in vivo [5], and the HRP reaction was performed on the fixed liver tissue and isolated vesicles.

A histochemical study of the HRP reaction in the liver tissue (Fig. 3A) shows that the electron-dense reaction product is detected in the space between the cells but not in the bile canaliculus. Endocytic vesicles filled with HRP bud off from the plasma membrane (Fig. 3B, arrow) or are present in the cells. As shown in Fig. 3C, the final preparation of the H^+-pump-rich membrane vesicles contains a number of vesicles that exhibit intravesicular staining of HRP. As the perfusion

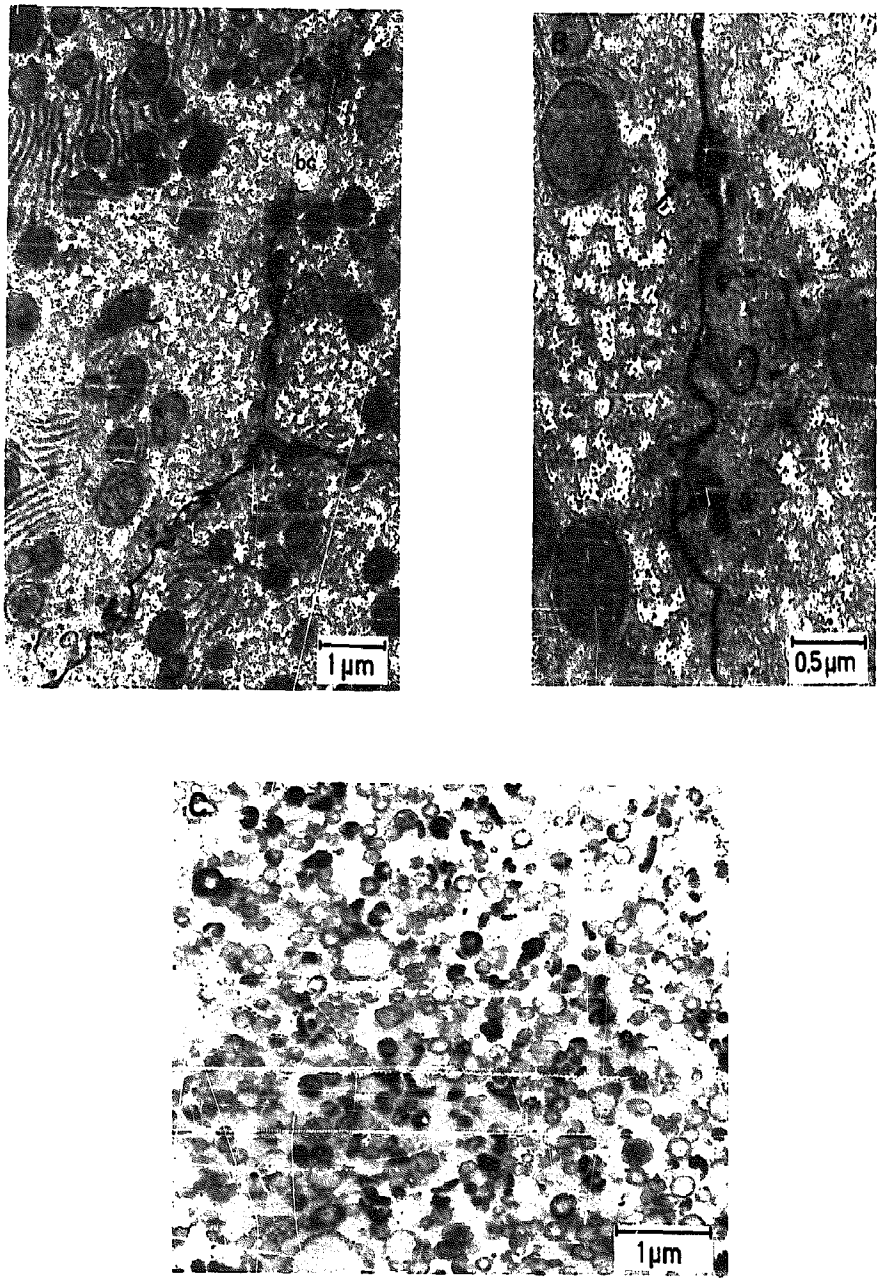

Fig. 3. Horseradish peroxidase activity (HRP) in rat liver tissue (A and B) and isolated endocytic vesicles (C). The enzyme was injected into a rat in vivo and 15 min later, the liver was removed, fixed and reacted for HRP (for details, see Materials and Methods). Endocytic vesicles were isolated from HRP-injected rats, fixed as pellet and then reacted for HRP demonstration. (A) This electron micrograph (×10880) depits the intercellular cleft between rat liver cells. The cleft is filled with the reaction product of HRP. The cell membranes display a number of invaginations from which endocytic vesicles originate. The bile canaliculus (bc) is not labeled. (B) Higher magnification (×64000) of a tortuous intercellular cleft labelled by HRP. The arrow points to a endocytic vesicle just forming. (C) A fraction of liver membranes enriched in endocytic vesicles, some of which are labeled by HRP (×16000).

TABLE I

YIELDS OF PROTEIN AND ATP-DRIVEN H^+-PUMP ACTIVITY DURING PREPARATION OF RAT LIVER ENDOCYTIC VESICLES

Values are means ± S.E. of four experiments. H^+-pump activity was measured by diluting an aliquot of homogenate or indicated fraction into KCl-buffer, which contained acridine orange ± ATP. The initial rates of fluorescence quenching in the absence of ATP were subtracted from the rates observed with ATP. S (supernatant), P (pellet), and PG (fraction from the Percoll gradient) are explained in Materials and Methods. EV, endocytic vesicles, ΔF, fluorescence change.

Fraction	Protein		H^+-pump		
	total (mg)	yield (%)	total activity (ΔF/total prot.)	yield (%)	specific activity (ΔF/mg protein)
Homogenate	4329 ±105.1	100	6530 ±914.7	100	1.51±0.22
S_1	2061 ± 96.7	47.6 ±1.23	5181 ±127.5	83.8 ±10.6	2.53±0.16
S_2	1649 ± 28.1	38.1 ±0.38	3135 ±111.5	51.9 ± 9.5	1.90±0.05
P_3	141.6 ± 11.2	3.28 ±0.25	1397 ±263.1	24.4 ± 8.2	9.87±1.57
PG_2	24.6 ± 0.99	0.57 ±0.024	294 ± 16.4	4.83± 0.82	12.0 ±0.85
P_4	11.0 ± 2.01	0.26 ±0.043	225 ± 21.3	3.79± 0.92	21.3 ±1.76
S_5	2.78± 0.17	0.064±0.003	124 ± 11.7	2.05± 0.39	44.7 ±3.74
EV	0.66± 0.11	0.015±0.003	67.5± 5.88	1.14± 0.25	106.0 ±9.17

time with HRP injected into rats in vivo was only 15 min, it was not expected that all endocytic vesicles in the cells had taken up HRP. Morphologically, the preparation of our vesicles is similar to the liver endosomal preparations described previously by other using different isolation techniques [5–9].

Table I summarizes the yield of protein and H^+-pump activity in the presence of Cl^- in the most important purification steps. The yield of protein in final vesicle preparations was 0.66 mg, i.e., only 0.015% of the starting amount of protein in the homogenate obtained from two livers. The yield of H^+-pump activity was about 1%, with a concomitant increase in the specific activity to about 100-fold. The total recovery of H^+-pump activity in all fractions varied between 85% and 105% (data not shown), ruling out any significant inactivation or activation of the pump during the purification procedure.

The purity of our endosomal preparations was estimated from the enrichment factors for the

TABLE II

ENRICHMENT FACTORS FOR H^+-PUMP AND VARIOUS ENZYMES IN RAT LIVER ENDOCYTIC VESICLES

Values are means ± S.E. for the number of preparations (n). Enrichment factor is the ratio of specific activity in the final preparation over that in the homogenate.

Enzyme (specific marker for)	n	Enrichment factor
H^+-pump (endocytic vesicles)	36	30.1 ±2.66
Succinate–cytochrome c oxidoreductase (mitochondria)	5	0.21±0.05
KCN-resistant NADH-oxidoreductase (endoplasmic reticulum)	5	0.20±0.06
Thiamin pyrophosphatase (endoplasmic reticulum+Golgi membranes)	4	1.26±0.22
Acid phosphatase (lysosomes)	4	3.49±0.28
Acid 5′-nucleotidase (lysosomal membranes)	4	2.83±0.36
β-Glucosaminidase (lysosomes)	4	0.42±0.05
$(Na^+ + K^+)$-ATPase (sinusoidal membranes)	6	2.31±0.44
Alkaline phosphatase (canalicular membranes)	5	6.44±0.46
Alkaline 5′-nucleotidase (canalicular membranes)	5	7.43±0.23
Leucine arylamidase (canalicular membranes)	8	3.85±0.14
Acetylcholinesterase (red blood cells)	5	0.81±0.09

TABLE III

CHARACTERISTICS OF H^+-PUMP IN LIVER ENDOCYTIC VESICLES

Dependence on nucleotides was measured by diluting 0.14 mg vesicle protein in KCl buffer which contained acridine orange and 1.5 mM of the indicated nucleotides. Dependence on monovalent cations was determined by diluting 0.065 mg vesicle protein into buffers which comprised 300 mM mannitol/5 mM $MgSO_4$/6 μM acridine orange/1.5 mM ATP/5 mM Hepes-Tris (pH 7.0), plus 125 mM of the indicated monovalent cations as chloride salts. Dependence on divalent cations was measured by diluting 0.19 mg vesicle protein into acridine orange, ATP and KCl-containing buffers, which comprised 5 mM of either Mg^{2+} or other indicated cations as chloride salts. Anion specificity was recorded after the vesicles (0.085 mg protein) had been added to buffers which contained 300 mM mannitol/100 mM potassium gluconate/5 mM $MgSO_4$/6 μM acridine orange/1.5 mM ATP/5 mM Hepes-Tris (pH 7.0), plus 25 mM of the indicated anions in form of potassium salts. The kinetics of H^+-pump were determined in buffers with various Cl^- concentrations. Osmolarity was kept constant by addition of gluconate. Kinetic characteristics were estimated from a Lineweaver-Burk plot of the data. TMA = tetramethylammonium. n = number of experiments.

Parameter	Result
Dependence on nucleotides	ATP (no activity with GTP, CTP, ITP, UTP)
Dependence on monovalent	$K^+ = TMA^+ = choline^+ = Na^+ = Li^+ = Rb^+ = Cs^+$
Dependence on divalent cations	$Mg^{2+} = Mn^{2+} > Co^{2+} \gg Cu^{2+}$ (no activity with Ca^{2+} and Zn^{2+})
Anion specificity	$I^- > Cl^- = Br^- \gg$ gluconate $= SCN^- = HCO_3^- = NO_3^- > F^- = SO_4^{2-} = PO_4^{3-}$
K_m (Cl^-)	23.1 ± 2.67 mM ($n = 4$)
V_{max}	86.3 ± 11.52 ΔF/min per mg protein ($n = 4$)

H^+-pump and enzymes known to be markers for various cellular structures. The enrichment factor for H^+-pump activity was determined from the initial rates of Cl^--stimulated H^+-pump activity in the liver homogenate and final vesicle preparations. As shown in Table II, the vesicles were enriched about 80-fold in H^+-pump activity. The activities of marker enzymes for mitochondria, endoplasmic reticulum, Golgi membranes and red blood cells were not enriched. A small enrichment, 3–4-fold, was found for the lysosomal markers, acid phosphatase and acid 5′-nucleotidase, but not for β-glucosaminidase. ($Na^+ + K^+$)-ATPase activity, a marker for sinusoidal membranes, was enriched about 2-fold. An enrichment of between 4- and 7-fold was observed for the activities of the putative membrane markers for bile canaliculi, alkaline phosphatase, alkaline 5′-nucleotidase and leucine arylamidase. The data indicate that liver endocytic vesicles isolated by our method are contaminated by canalicular membranes, but are devoid of a significant contamination by other cellular membranes.

Characterization of the H^+-pump

Besides the sensitivity to various inhibitors demonstrated in Fig. 1, the H^+-pump in our endosomal preparation was further characterized with respect to cation and anion specificity, electrogenicity and ATPase activity. The experimental approach was the same as described in details previously for rat renal cortical endocytic vesicles [12]. The original recordings are not shown, the conclusions are summarized in Table III.

The endosomal-H^+-pump shows a high activity with ATP and no activity with other nucleotides. A requirement for a specific monovalent cation was not observed. However, the pump required the divalent cations Mg^{2+} or Mn^{2+}. The activity was about half-maximal with Co^{2+} and very small with Cu^{2+}. No activity was measured with Ca^{2+} or Zn^{2+}. As for the anions, the highest activity was recorded with I^-, followed by Cl^- and Br^-. Much smaller H^+ uptake was found with gluconate, SCN^-, HCO_3^-, and NO_3^-. F^-, SO_4^{2-} and PO_4^{3-} sustained the H^+-pump activity less well than gluconate. When measured at various concentrations of Cl^-, the H^+-pump obeyed hyperbolic kinetics, exhibiting a half-maximal activity at 23 mM chloride and a V_{max} of about 86 ΔF/min per mg protein.

The electrogenicity of the H^+-pump was studied by fluorescence changes of Oxonol V (Fig. 4). In the absence of Cl^- (Fig. 4A, KGluc), ATP caused a quenching of dye fluorescence, indicating the development of an inside-positive membrane potential created by the ATP-dependent H^+-pump. The membrane potential created dissipated by

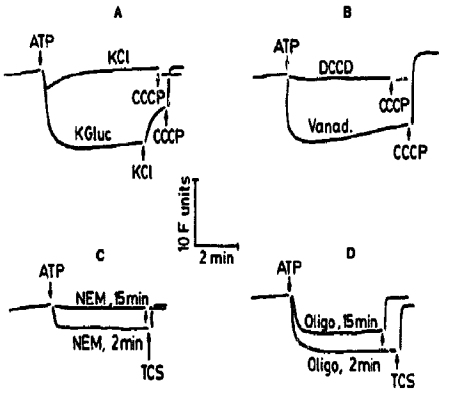

Fig. 4. Electrogenicity of the H^+-pump. Vesicles (0.259 mg protein), preloaded with potassium gluconate buffer were added to the same (KGluc and all other recordings except that designated as KCl) or KCl buffer (KCl) which contained Oxonol V and one of the indicated inhibitors in the concentrations listed in the legend of Fig. 2. The control experiment (KGluc) contained 0.5% ethanol. The vesicles were preincubated with the inhibitors or ethanol at room temperature for 2 or 15 min. The development of the membrane potential was started by adding ATP (final concentration 1.5 mM). At the indicated time (arrows), either KCl (final concentration, 25 mM) or a protonophore (CCCP or TCS, each in final concentration of 5 μM) were added to dissipate the membrane potential created.

adding Cl^- or the protonophore, CCCP. In a Cl^--containing buffer (Fig. 4A, KCl), the development of a membrane potential was prevented, indicating that Cl^- acts as a permeant, charge-compensating anion. In accordance with FITC-dextran and acridine orange studies shown in Fig. 2, the development of membrane potential by the ATP-driven H^+-pump in liver endosomes was unaffected by vanadate (Fig. 4B), slightly inhibited by oligomycin (Fig. 4D), and strongly inhibited by DCCD (Fig. 4B) and NEM (Fig. 4C).

ATPase activity

ATPase activity in isolated vesicles was measured by the coupled optical assay described previously [12]. In a Cl^--free buffer and in the presence of 2 mM ouabain, 5 μg/ml oligomycin, and 1 mM levamisole, the vesicles exhibited an ATPase activity (in nmol ATP/min per mg protein) of 7.14 ± 0.40 ($n = 5$) at 37°C. This ATPase activity was negligible stimulated by Cl^- (7.45 ± 0.36, $n = 6$). A stimulation by CCCP (7.32 ± 0.25, $n = 6$) and an inhibition by vanadate were not observed (7.03 ± 0.33, $n = 6$). DCCD and NEM inhibited significantly (5.39 ± 0.47, $n = 6$, and 5.98 ± 0.37, $n = 6$, respectively; vs. controls, $P < 0.05$).

Discussion

By using a high-speed differential centrifugation and a single Percoll density gradient, a vesicle fraction was isolated from rat liver homogenate that exhibits a high ATP-driven, anion-stimulated H^+-pump activity. The endosomal origin of these vesicles is concluded from the following findings: (a) the vesicles had taken up the fluid-phase markers FITC-dextran and HRP, injected into rats in vivo, as also shown for endosomes in other cells [4,5,26]; (b) the vesicles showed an enrichment in H^+-pump of about 80-fold, whereas the contamination by marker enzymes for other cellular membranes was relatively small. A 3- to 4-fold enrichment in lysosomal markers, acid phosphatase and acid 5'-nucleotidase, may indicate a partial contamination of our vesicle preparations with lysosomes. However, the negligible enrichment of the third lysosomal enzyme, β-glucosaminidase, indicates that not intact lysosomes but rather lysosomal membranes or detached enzymes from lysosomes are found in our preparation [19,27]. Other significant contaminants are bile canalicular membranes, since the enrichment factors for the respective marker enzymes vary between 3 and 8. However, since bile canalicular membranes do not take up in vivo injected FITC-dextran, the characterization of the H^+-pump with trapped dye is not influenced by this contamination. Furthermore, it has to be emphasized that the contamination of our vesicles with Golgi membranes is quite low (1.2-fold enrichment in thiamin pyrophosphatase), whereas the majority of preparations of liver endosomes described by others deals with a considerable enrichment in marker enzymes for Golgi membranes [7–9,25]; (c) the morphological appearance of our endosomal preparations was remarkably similar to the appearance of endocytic vesicles isolated by other methods [5–9]; (d) finally, the H^+-pump in vesicles, measured by the fluorescence changes of either intravesicularly trapped FITC-dextran or acridine orange, exhibited all the characteristics of the vacuolar type H^+-pumps previously demon-

strated in endocytic vesicles from various cells, including liver [3,4,9,12], and in other components of the vacuolar system [3,28]. As shown here, this type of H^+-pump prefers ATP, depends on divalent cations, such as Mg^{2+} or Mn^{2+}, but not on monovalent cations, is stimulated by several anions, such as I^-, Cl^-, and Br^-, is electrogenic, and is resistant to ouabain and vanadate, weakly inhibited by oligomycin, and strongly inhibited by DCCD and NEM. In accordance with the findings in fluorescence studies, our endosomes also exhibit an oaubain-, vanadate- and levamisole-insensitive ATPase activity, which is inhibited by DCCD and NEM, indicating the presence of an H^+-ATPase in the vesicles membranes.

As a similar method of isolation has been applied to enrich endocytic vesicles from rat renal cortex [4] and to study their H^+-pump [12], it is interesting to compare the characteristics of the vesicles and the H^+-pumps in renal and liver endosomal preparations. Besides many similarities, there are two important differences. On a 16% wt/wt Percoll density gradient, the renal endocytic vesicles appear at the bottom, whereas the liver endosomes peak in the top fractions, indicating the presence of tissue differences in endosomal densities. The low density of liver endosomal vesicles has been reported previously [5–8]. Tissue differences are further supported by the studies on anion dependence of the H^+pump. The H^+-pump in renal endocytic vesicles is best stimulated by SCN^-, followed by Cl^-, Br^- and I^-. The stimulation of the pump in liver endosomes is highest with I^-, followed by Cl^- and Br^-. The activity of the H^+-pump in the presence of SCN^- was rather small, and not different from that in the presence of gluconate. As described previously [29], renal endocytic vesicles possess a conductive anion path that accepts Cl^-, SCN^-, Br^- and I^-, but not other monovalent and divalent anions. In liver endosomes, this anion conductance seems to be even more selective and does not accept other anions, except for halides.

In conclusion, a simple and efficient high-speed centrifugation method is described for the isolation of endocytic vesicles from mammalian liver. The experiments confirmed that these vesicles contain an electrogenic ATP-driven vacuolar-type H^+-pump.

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References

1 Steinman, R.M. Mellman, I.S., Muller, W.A. and Cohn, Z.A. (1983) J. Cell Biol. 96, 1–27.
2 Wileman, T., Harding, C. and Stahl, P. (1985) Biochem. J. 232, 1–14.
3 Mellman, I., Fuchs, R. and Helenius, A. (1986) Annu. Rev. Biochem. 55, 663–700.
4 Sabolić, I., Haase. W. and Burckhardt, G. (1985) Am. J. Physiol. 248, F835–F844.
5 Courtoy, P.J., Quintart, J. and Baudhuin, P. (1984) J. Cell. Biol. 98, 870–876.
6 Wall, D.A. and Hubbart, A.L. (1985) J. Cell Biol. 101, 2104–2112
7 Evans, W.H. and Flint, N. (1985) Biochem. J. 232, 25–32.
8 Khan, M.N., Savoie, S., Bergeron, J.J.M. and Posner, B.I. (1986) J. Biol. Chem. 261, 8462–8472.
9 Marsh, M., Schmid, S., Kern, H., Harms, E., Male, P., Mellman, I. and Helenius, A. (1987) J. Cell. Biol. 104, 875–886.
10 Dickson, R.B. Beguinot, L., Hanover, J.A., Richert, N.D., Willingham, M.C. Pastan, I. (1983) Proc. Natl. Acad. Sci. USA 80, 5335–5339.
11 Saemark, T., Flint, N. and Evans W.H. (1985) Biochem. J. 225, 51–58.
12 Sabolić, I. and Burckhardt, G. (1986) Am. J. Physiol. 250, F817–F826.
13 Graham, R.C. and Karnowsky, M. (1966) J. Histochem. Cytochem 14, 291–302.
14 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
15 Berner, W. and Kinne, R. (1976) Pfluegers Arch. 361, 269–278.
16 Fleischer, S. and Fleischer, B. (1967) Methods Enzymol. 10, 406–432.
17 Sottocasa, G.L., Kuylenstierna, L., Ernster, L. and Bergstrand, A. (1967) J. Cell Biol. 32, 415–438.
18 Scalera, V., Storelli-Joss, C., Haase, W. and Murer, H. (1980) Biochem. J. 186, 177–181.
19 Burnside, J. and Schneider, D.L. (1982) Biochem. J. 204, 525–534.
20 Novikoff, A.B. and Heus, M. (1963) J. Biol. Chem. 238, 710–716.
21 Fiske, C.H. and SubbaRow, Y. (1925) J. Biol. Chem. 66, 375–388.
22 Sabolić, I. and Burckhardt, G. (1983) Biochim. Biophys. Acta 734, 210–220.
23 Van Dyke, R.V., Scharschmidt, B.F. and Steer, C.J. (1985) Biochim. Biophys. Acta 812, 423–436.
24 Van Dyke, R.W., Hornick, C.A., Belcher, J., Scharschmidt, B. and Havel, R.J. (1985) J. Biol. Chem. 260, 11021–11026.

25 Quintart, J., Courtoy, P.J. and Baudhuin, P. (1984) J. Cell. Biol. 98, 877–884.

26 Yamashiro, D.J., Fluss, S.R. and Maxfield, F.R. (1983) J. Cell Biol. 97, 929–934.

27 Biber, J., Stieger, B., Haase, W. and Murer, H. (1981) Biochim. Biophys. Acta 647, 169–176.

28 Schenkenfeld, B.F. and Van Dyke, R.W. (1987) Annu. Rev. Physiol. 49, 69–83.

29 Burckhardt, G., Meyers, B. and Sabolic, I. (1987) in Molecular Nephrology, Biochemical Aspects of Kidney Function (Kovacevic, Z. and Guder, W.G. eds.), pp. 37–62, De Gruyter, Berlin, New York.