Ethinyl Estradiol Decreases Acidification of Rat Liver Endocytic Vesicles

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Treatment with ethinyl estradiol is known to impair bile formation, bile acid transport and Na,K-ATPase activity, to alter receptor-mediated endocytosis and transcytosis of IgA and asialoorosomucoid and to affect membrane lipid composition and fluidity. Because appropriate sorting and trafficking of asialoorosomucoid requires adequate acidification of endocytic vesicles by a lipid-sensitive electrogenic proton pump, we examined the effects of 5 days of treatment with ethinyl estradiol (5 mg/kg body wt, subcutaneously) on acidification of early endosomes prepared from male rat livers. Littermate control animals received equal volumes of the solvent propylene glycol. Pretreatment with ethinyl estradiol reduced ATP-dependent initial rates of endosome acidification by 11% to 25% when measured in potassium medium containing 0 to 140 mmol/L chloride; these differences were significant at four of six chloride concentrations tested. The proton pumps of ethinyl estradiol and propylene glycol endosomes exhibited similar Michaelis-Menten constants for MgATP (Michaelis-Menten constant of 63 and 66 μmol/L in the absence of chloride and 101 and 126 μmol/L in the presence of chloride, respectively). Acidification of ethinyl estradiol and propylene glycol endosomes changed in the same manner when various cations or anions were substituted for potassium gluconate, although the effects of ethinyl estradiol were less marked in the absence of K^+. Kinetics of inhibition for ethinyl estradiol and propylene glycol endosomes were similar for the proton pump inhibitors N-ethylmaleimide (50% inhibitory concentrations of 13.5 and 18.1 μmol/L), dicyclohexylcarbodiimide (50% inhibitory concentrations of 206 and 216 μmol/L) and bafilomycin A (50% inhibitory concentrations of 11 and 6 nmol/L). Although initial rates of acidification were slower in ethinyl estradiol endosomes, ATP-dependent steady-state vesicle interior pH was the same as that of propylene glycol endosomes over a range of chloride concentrations; this appeared to be due mainly to a trend toward decreased proton leak rates in ethinyl estradiol endosomes. Overall, ethinyl estradiol treatment modestly decreased initial rates of acidification and vesicle proton leakage, perhaps because of changes in endosome lipid composition; differences in the number, density or activation state of proton pumps; or differences in endosome geometry. Because the decrease in acidification rates was small, the effects of estrogen on the efficiency of uncoupling of endocytosed ligands such as asialoorosomucoid from their receptors in early endosomes; thus the rates of sorting and distribution of ligands remain unclear. (HEPATOLOGY 1993;18:604-613.)

Endocytic vesicles and other intracellular organelles contain an electrogenic proton pump or vacuolar H^+-ATPase (1-3) that appears to function in parallel with a chloride conductance (3-7) to maximally acidify the interiors of these vesicles. This interior acidic pH (pH_i) is essential for normal receptor-mediated endocytosis and degradation of macromolecules (2, 3, 8-11) and processing, sorting and secretion of some proteins from the Golgi apparatus (10). Changes in vesicle acidification may alter these cell functions (8-10), but little is known about mechanisms for regulating vesicle acidification *in vivo*. The liver is very active in the receptor-mediated endocytosis and degradation of lipoproteins and asialoglycoproteins and exhibits an active transcytotic pathway from sinusoidal blood to bile (11, 12). Therefore it is a good model for studying the relationship between changes in vesicle acidification and the sorting and degradation of macromolecules.

Ethinyl estradiol (EE) treatment is known to inhibit hepatic bile formation (13-19) and bile acid transport (17-19) and to affect ion pumps such as Na,K-ATPase (13-19) and Ca^{+2}-ATPase (20). Previous investigators have shown that in rat liver this agent alters sorting of asialoglycoprotein asialoorosomucoid (ASOR) subjected to endocytosis and reduces transcytotic movement of IgA from blood to bile (12). Because correct sorting, delivery and degradation of asialoglycoproteins requires adequate vesicle acidification, we undertook these studies to determine whether EE altered acidification of

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the early rat liver endosomes involved in sorting and delivery of protein ligands to lysosomes.

MATERIALS AND METHODS

Materials. All chemicals except ATP (Boehringer Mannheim, Indianapolis, IN) and fluorescein antibody (Molecular Probes, Inc., Eugene, OR) were purchased from Sigma Chemical Co. (St. Louis, MO). Bafilomycin A_1 was the kind gift of Dr. Karlheinz Altendorf (University of Osnabruck, Osnabruck, Germany); it was prepared as a stock solution in DMSO. Carbonyl cyanide m-chlorophenylhydrazone (CCCP), dicyclohexylcarbodiimide (DCCD) and monensin were prepared as stock solutions in ethanol. N-ethylmaleimide (NEM) was prepared daily as a stock solution in distilled water. Before use 70,000 Da FITC-conjugated dextran (FITC-dextran) was purified by dialysis for 4 or 5 days against 20 mmol/L phosphate buffer (pH 7.0), lyopholized and stored at −20° C.

Animal Preparation. Rats were housed and treated in accordance with a protocol approved by the University of Michigan Committee on the Care and Use of Animals in accordance with the guidelines outlined by the Institute of Laboratory Animal Resources and the National Institutes of Health. For these studies, male Sprague-Dawley rats were treated with EE (5 mg/kg body wt, subcutaneously) for 5 days. Control animals were littermates given identical volumes of the solvent propylene glycol (PG). Animals were purchased, housed and treated as pairs (one animal given EE and the other PG), and endosomes were prepared and assayed simultaneously each day from an EE-treated animal and its PG-treated littermate control.

Isolated Endosomes. Early (10-min) endosomes were prepared as a microsomal pellet from male Sprague-Dawley rat liver 10 min after intravenous injection of 50 mg 70,000 Da FITC-dextran dissolved in 1 ml normal saline solution. Livers were rapidly chilled by perfusion with ice-cold sucrose buffer (250 mmol/L sucrose and 3 mmol/L imidazole, pH 7.4), chopped into small pieces (1 to 2 mm) with scalpels and homogenized in 5 vol of ice-cold sucrose buffer by six passes of a loose-fitting Dounce homogenizer and one pass of a tight-fitting Dounce homogenizer. Homogenates were centrifuged at 1,000 $g_{average}$ for 10 min to remove nuclei and unbroken cells. Supernatants were removed and centrifuged at 10,000 $g_{average}$ for 20 min. Supernatants were removed, and a microsomal pellet that contained low-density fluorescent endosomes was prepared by centrifugation at 100,000 $g_{average}$ for 60 min. Pellets were resuspended in a small volume of ice-cold potassium gluconate buffer (140 mmol/L potassium gluconate and 30 mmol/L *bis*[2-hydroxyethyl]imino-tris[hydroxymethyl] methane [Bis-Tris], pH 7.0, with piperazine-N-N′-*bis*[2-ethane sulfonic acid] [PIPES]) with a small, loose Dounce homogenizer. For some studies, endosomes were resuspended in a similar buffer in which N-methyl-D-glucamine (NMDG) replaced K^+. All endosomes were used within 6 hr of preparation, and EE and PG endosomes were assayed alternately to eliminate time-dependent variations in ion transport.

Vesicle Acidification. Vesicle pH (pH_i) was assessed on the basis of the ratio of fluorescein fluorescence at two excitation wavelengths (excitation = 493 nm and 450 nm; emission = 530 nm) and a standard curve relating pH_i to fluorescence ratio as measured with a computer-controlled dual-excitation wavelength spectrofluorometer (SPEX Industries, Edison, NH) as described previously (21-24). Experiments were performed at 22° C. For each assay, 500 to 700 μg endosomes were added to acrylic cuvettes containing 2 ml isotonic buffer (a mixture of 140 mmol/L KCl and 140 mmol/L potassium gluconate yielding the desired chloride concentration or isotonic amounts of other salts with 30 mmol/L Bis-Tris [pH 7.0]) 5 min before use with other additions as indicated in figure legends and tables. For all studies, cuvettes also contained sufficient fluorescein antibody to quench fluorescence of extravesicular fluorescein. To initiate acidification, we added 5 mmol/L MgATP. After we achieved steady state, we added proton ionophores (8 μl of an ethanol stock of 5 mmol/L monensin and 2 mmol/L CCCP) to eliminate all pH gradients, and a standard curve relating pH to fluorescence ratio was determined. Over the range of pH employed in these studies, the ratio of fluorescein fluorescence at 493 nm/450 nm was linearly related to pH. Each plot of fluorescence ratio vs. time was converted to a plot of pH_i vs. time with the standard curve. Initial rates of ATP-dependent acidification were calculated from the slope of a line, fit by eye, of vesicle pH_i vs. time, and steady-state pH_i was taken as the lowest stable value obtained (Fig. 1). This method for measuring acidification rate is independent of the number of endocytic vesicles present in the assay cuvette and the concentration of FITC-dextran in each endocytic vesicle (21-24; Van Dyke RW, Unpublished observations, 1990-1992). Indeed, in preliminary studies using these 10-minute endosomes, we confirmed that initial rates of acidification were independent of the amount of protein used in the assay (data not shown).

Plots of initial rates of acidification vs. medium concentration of MgATP or chloride were fitted, by non-linear curve-fitting (Kaleida Graph 2.1; Synergy Software, Reading, PA), on a Macintosh IIcx personal computer (Apple Computers), to functions of the form $y = ax/(b + x) + c$, and estimates for the constants were obtained. This function was selected because it best fitted the data and represents a form of a Michaelis-Menten function.

For statistical comparison of the kinetic parameters of 50% of effective dose (ED_{50}) and maximum velocity (V_{max}) for the acidification rate vs. chloride concentration curves, data from five paired preparations of EE and PG endosomes in which a range of chloride concentrations were tested were each fitted to this function. Estimates of ED_{50} and V_{max} obtained from these five separate preparations were then averaged, compared with paired Student's *t* tests and used to generate the overall curves shown in Figure 2.

Proton Leak Rates. Proton leak rates were measured as the initial linear rate of realkalinization of vesicles after the proton pump was stopped. Endosomes were allowed to acidify to steady state by addition of 3 mmol/L MgATP. Then ATP was rapidly consumed by addition of 15 mmol/L glucose and 15 to 25 units of type F-300 yeast hexokinase, and the initial linear rate of realkalinization was assessed as change in pH per minute (ΔpH/min). Preliminary studies showed that these amounts of glucose and hexokinase gave maximal rates of alkalinization. Small volumes (5 μl) of ethanol or valinomycin (1 mmol/L) dissolved in ethanol were added concomitantly with glucose and hexokinase.

Proton Fluxes. Influx and efflux (J_{H^+}) of protons in vesicles loaded with FITC-dextran were calculated as the product of measurements of vesicle buffering capacity *(B)* (millimoles per pH) and the initial rates of ATP-dependent acidification or realkalinization after the proton pump was stopped: $J_{H^+} = B \cdot \Delta pH/min$.

Buffering capacity was measured with a modification of an ammonium-pulse technique (22, 23). In these studies, B was measured at two values of pH_i. We have previously shown that buffering capacity in endocytic vesicles increases with pH_i (22, 23). For calculation of pump-mediated proton influx rates, we used linear-regression analysis to extrapolate B for pH_i values

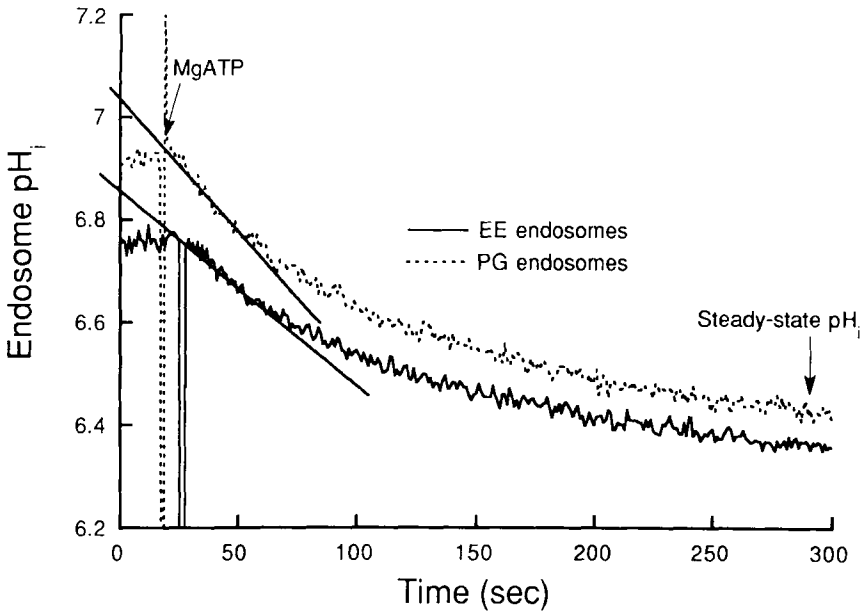

FIG. 1. Representative endosome acidification assays. FITC-dextran–loaded endosomes were prepared from EE- and PG-treated rats as described in Materials and Methods and assayed in 140 mmol/L potassium gluconate buffer. Endosomal pH_i was assessed from the ratio of fluorescein fluorescence at two excitation wavelengths and a standard curve. Rates of acidification were calculated as the slopes of tangents (——) drawn to the initial portion of the acidification curve after addition of MgATP. Steady-state ATP-dependent pH_i was taken as the value of pH_i observed before addition of monensin. In this example, the initial rates of acidification were 0.224 ΔpH/min (EE) and 0.295 ΔpH/min (PG). Curves were offset for clarity.

at the point at which MgATP was added. For calculation of proton permeabilities, we used the value of B measured at the lowest pH_i tested (5.99 for EE endosomes and 5.91 for PG endosomes).

Vesicle proton permeability *(P)* was calculated from the proton efflux rate as

$$P(10^3 \cdot \text{min}^{-1}) = J_H \cdot (\text{mmol} \cdot \text{min}^{-1})/[H^+_i] - [H^+_o])$$

where $[H^+_i]$ and $[H^+_o]$ are intravesicular and extravesicular proton concentrations, respectively (22, 23, 25).

Other Considerations. Protein concentrations were measured with the method of Lowry et al. (26).

Statistics. Experiments were repeated using two to 14 different preparations of vesicles; results were expressed as absolute values or as a percentage of results in concurrent controls. Results were expressed as the mean ± S.E.M. ($n \geq 3$) of results from different numbers of preparations. Where the sample size was at least three, experimental values were compared with control values with paired Student's *t* tests. A p value less than 0.05 was considered statistically significant.

RESULTS

Endosomes prepared from rats treated with EE or PG exhibited ATP-dependent acidification (Fig. 1). Although endosomes used in this study were not purified, only vesicles formed during the 10-min exposure to FITC-dextran contained internalized fluorescein and contributed to measurement of pH_i. Small amounts of free FITC-dextran, released from vesicles during homogenization and centrifugation, were sometimes present in assay cuvettes, but the fluorescence of this material was completely quenched by addition of fluorescein antibody (data not shown; 22, 23) before addition of MgATP. Incubation of endosomes with MgATP and other agents at 22° C did not result in release of additional FITC-dextran over the course of these studies (data not shown).

In five paired preparations of endosomes, EE treatment (Fig. 2) consistently decreased the initial rate of MgATP-dependent acidification by 11% to 25% compared with PG treatment (25%, 17.5%, 16.7%, 14.5%, 10.7% and 11% in 0, 2, 5, 10, 25, 140 mmol/L Cl^-, respectively); these differences were statistically significant for assays in 0 ($p < 0.005$), 2 ($p < 0.05$), 25 ($p < 0.005$) and 140 ($p < 0.05$) mmol/L chloride. As noted by us in other intracellular acidified vesicles, endosome acidification was increased by increases in medium chloride concentration; this effect was probably related to neutralization of the positive charge of pumped protons by the permeable anion chloride. When the data were fit to a Michaelis-Menten–type function, estimates for the ED_{50} of chloride were similar for EE (3.3 ± 0.3 mmol/L) and PG (3.3 ± 1.3 mmol/L) (p = NS) endosomes, whereas V_{max} was decreased by 11.2% in EE endosomes (0.924 ± 0.053 ΔpH/min vs. 1.033 ± 0.054 ΔpH/min) ($p \leq 0.05$). These findings suggest that EE endosomes have fewer proton pumps per vesicle or the same density of proton pumps, which turn over more slowly than proton pumps in PG endosomes.

Other explanations for the differences in acidification rates may include differences in vesicle buffering ca-

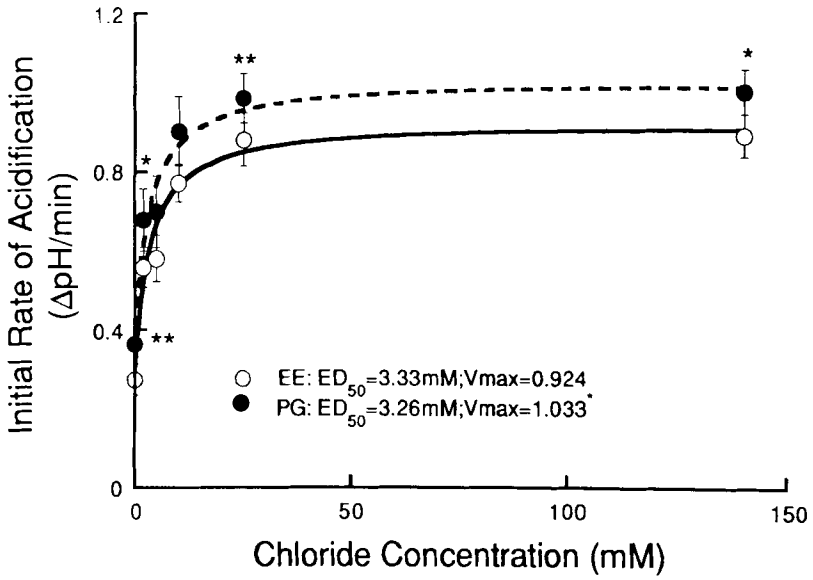

FIG. 2. Initial rates of ATP-dependent acidification of endosomes prepared from rats treated with EE (○) or PG (●) plotted against medium chloride concentration. Vesicles were suspended in medium containing 140 mmol/L K^+ with a mixture of gluconate and chloride to give the indicated [Cl^-] and 30 mmol/L Bis-Tris (pH 7.0). Symbols represent mean ± S.E.M. for results from five different paired preparations of EE and PG endosomes. *$p < 0.05$ compared with PG; **$p < 0.005$ compared with PG. ED_{50} and V_{max} values were estimated from the best-fit function of the form $y = ax/(b + x) + c$ as described in Materials and Methods for each of the five different paired preparations of endosomes, and mean values for ED_{50} and V_{max} were calculated. The curve illustrated in this figure represents those averaged parameters.

pacity. We measured buffering capacity of acidified EE and PG endosomes with the ammonium-pulse technique in KCl and in potassium gluconate medium. As shown in Table 1, buffering capacity increased with more alkaline pH_i, as we have previously observed for purified rat liver "late" endosomes and lysosomes (22, 23). EE endosomes exhibited buffering capacities similar to but slightly lower than those of PG endosomes; values shown in Table 1 were similar to values we measured previously in 10-min endosomes prepared from untreated male Sprague-Dawley rats (23). Estimated values for B at the pH_i where initial rates of acidification were measured were similar for EE and PG endosomes in KCl (89.2 mmol/ΔpH and 88.2 mmol/ΔpH, respectively) and in potassium gluconate (90.2 mmol/ΔpH and 93.1 mmol/ΔpH, respectively) media. Therefore the total ATP-dependent proton influx rates (J_{H^+}) in EE and PG endosomes exhibited differences analogous to those shown in Figure 2. J_{H^+} for EE endosomes was 79 mmol/min (25 mmol/L KCl) and 25 mmol/min (potassium gluconate); for PG endosomes it was 87 mmol/min (25 mmol/L KCl) and 34 mmol/min (potassium gluconate).

To further explore the mechanism by which EE altered endosome acidification, we examined the kinetic response of endosome acidification to changes in the concentration of MgATP, the energy source for the proton pump. As shown in Figure 3, EE treatment did not affect the apparent affinity of the proton pump for MgATP in the presence or absence of chloride. Apparent K_m values for MgATP, estimated from the functions shown in Figure 2, were, in potassium gluconate medium, 67 μmol/L (EE) and 64 μmol/L (PG); in KCl medium they were 102 μmol/L (EE) and 126 μmol/L (PG). Thus EE did not appear to alter the catalytic structure of the proton pump.

TABLE 1. Buffering capacities of endosomes

Buffer ion	pHi[a]	EE (mmol/pH)[b]	pH_i	PG (mmol/pH)[b]
KCl	5.99	40.8 ± 1.2	5.91	42.8 ± 1.4
Potassium gluconate	6.19	57.7 ± 2.2	6.16	58.8 ± 2.2

[a]pH_i indicates intravesicular pH at the time NH_4Cl was added to assess buffering capacity.

[b]Values expressed as mean ± S.E.M. of assays performed in duplicate in four separate pairs of endosome preparations.

The endosome proton pump is electrogenic, and rates of acidification of endocytic vesicles are determined in part by the presence of relatively permeable charge-neutralizing anions or cations that reduce pump-generated membrane potential ($\Delta\Psi$) and increase ΔpH/min (22, 23, 27). Because EE could affect permeability of counteranions or cations, we examined the response of acidification rates of EE and PG endosomes to replacement of potassium and gluconate with a variety of anions and cations (Table 2). As we have shown previously for other endocytic vesicles (23, 27), anion replacement substantially altered acidification rates of both EE and PG endosomes in the sequence Cl^-, Br^- > gluconate > NO_3^-, PO_4^{-2} > SO_4^{-2}. The reduced acidification rates seen with the bulky anions PO_4^{-2} and SO_4^{-2} and with the usually more permeable NO_3^- (27) may reflect poor permeability and modest direct inhibitory effects on the proton pump (3, 27). Cation substitution had little effect as long as chloride

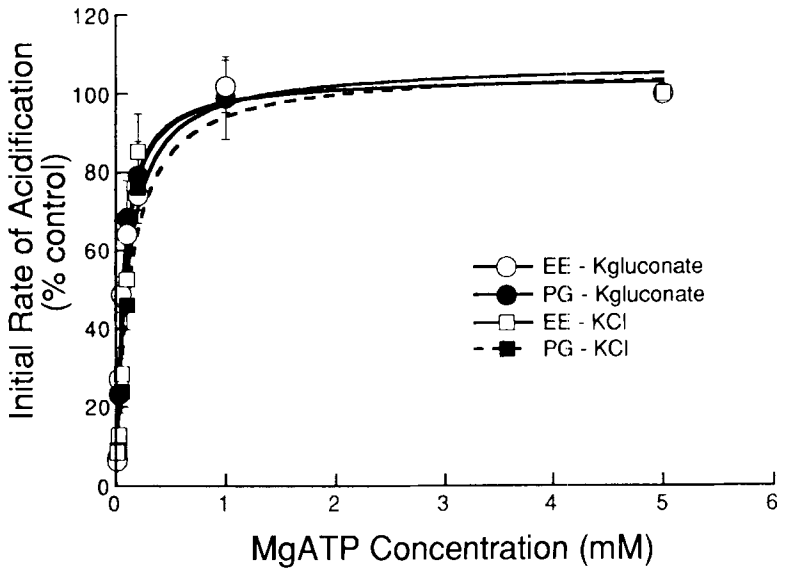

FIG. 3. Effect of changes in MgATP concentration on initial rates of acidification of EE-treated and PG-treated endosomes. Endosomes were resuspended and assayed in buffer containing 30 mmol/L Bis-Tris (pH 7.0) and 140 mmol/L potassium gluconate *(circles)* or 140 mmol/L KCl *(squares)*. Data expressed as percentages of values obtained in experiments performed on the same day using the same endosome preparation after addition of 5 mmol/L MgATP. Symbols represent the mean ± S.E.M. ($n \geq 3$) of results from two or three different paired preparations of endosomes. K_m values for MgATP were estimated, as described in Materials and Methods, as 67 μmol/L (EE, potassium gluconate), 64 μmol/L (PG, potassium gluconate), 102 μmol/L (EE, KCl) and 126 μmol/L (PG, KCl).

TABLE 2. Effect of ion substitution on initial rates of acidification

Ion	Concentration (mmol/L)	EE (% of 140 mmol/L potassium gluconate)	PG (% of 140 mmol/L potassium gluconate)
KCl	140	246	278
KBr	140	221	276
KNO_3	140	90	55
K_2HPO_4/sucrose	70/70	70	94
K_2SO_4/sucrose	70/70	54	49
NaCl	140	268	313
LiCl	140	439	404
Tetramethylammonium chloride	140	386	307
NMDG chloride	140	223	321
NMDG gluconate	140	37	33

Endosomes prepared from EE-treated or PE-treated rats were suspended in 140 mmol/L postassium gluconate buffer. Aliquots (25 μl) of endosomes were added to 2 ml buffer of the indicated composition and incubated at 22° C for 5 min. Initial rates of acidification were assessed and compared with rates measured in 140 mmol/L potassium gluconate buffer. Results are the mean of two separate paired preparations of endosomes.

was present; however, replacement of K^+ with the presumably less permeable cation $NMDG^+$ did substantially reduce acidification rates in gluconate medium. These findings suggest that EE and PG endosomes exhibit considerable permeability for Cl^- and Br^- and some permeability for K^+. Our findings also suggest that EE treatment does not generally alter cation or anion permeabilities.

The inhibitory effects of EE were also apparent in potassium-free medium. In three paired preparations, EE-treated and PG-treated endosomes were resuspended in NMDG gluconate buffer (140 mmol/L NMDG gluconate and 30 mmol/L Bis-Tris [pH 7.0]) and were assayed in NMDG buffers containing various concentrations of gluconate and Cl^-. Overall, PG endosomes in NMDG medium containing low Cl^- acidified at lower rates than did PG endosomes in K^+ medium containing equivalent amounts of Cl^-, especially in low-Cl^- medium. (0.265 vs. 0.356 ΔpH/min in the absence of Cl^-, 0.325 vs. 0.679 ΔpH/min in the presence of 2 mmol/L Cl^- and 0.856 vs. 0.943 ΔpH/min in 140 mmol/L Cl^-, respectively). In NMDG medium, EE endosomes acidified at rates 10%, 2%, 8% and 18% lower than those of PG endosomes in the presence of 0, 2, 25 and 140 mmol/L Cl^-, respectively. None of these differences were statistically significantly different. The extent to which EE treatment inhibited acidification in $NMDG^+$ medium was somewhat less than what we observed in K^+ medium (Fig. 2). This could reflect subtle effects of EE on K^+ permeability or a less dominant effect of EE

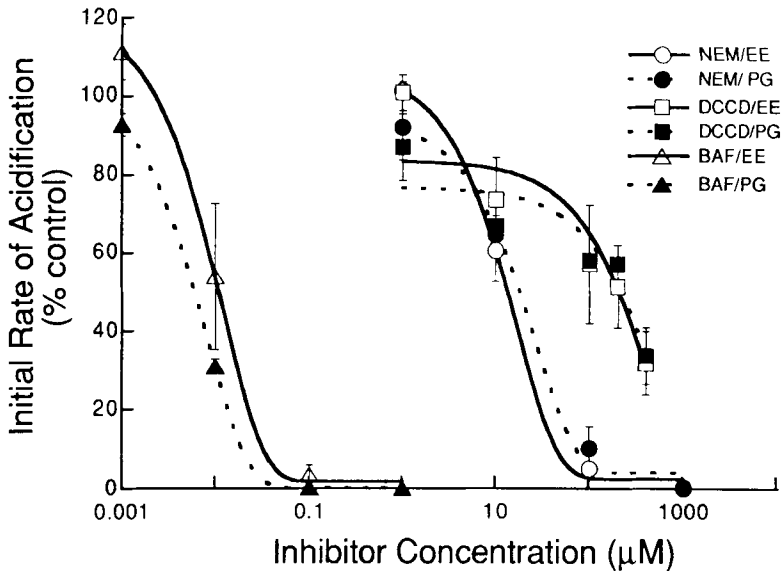

FIG. 4. Effect of proton pump inhibitors on initial rates of acidification of EE-treated and PG-treated endosomes. Endosomes were preincubated for 5 min in 25 mmol/L KCl, 115 mmol/L potassium gluconate, 30 mmol/L Bis-Tris (pH 7.0) buffer with the indicated concentrations of inhibitors before acidification was initiated by addition of 5 mmol/L MgATP. Results are expressed as percentages of control rates obtained in the same endosome preparation on the same day in assays containing equal volumes of solvent (ethanol for DCCD and NEM, DMSO for bafilomycin A_1). Curves represent best-fit functions of the form $y = ae^{(-bx)} + c$ (NEM, Bafilomycin A_1) or $y = ae^{(-bx)}$ (DCCD). We calculated fifty-percent inhibitory dose values from the estimated functions: NEM (○, ●), EE 13.5 μmol/L and PG 18.1 μmol/L; DCCD (□, ■), EE 206 μmol/L and PG 216 μmol/L; bafilomycin A_1 (△, ▲), EE 11 nmol/L and PG 6 nmol/L. Symbols represent the mean ± S.E.M. of results from three different paired preparations of endosomes.

on acidification when acidification was severely rate-limited by $\Delta\Psi$.

To investigate further possible structural or kinetic differences in the proton pumps of EE and PG endosomes, we evaluated the inhibitory profiles of three proton pump inhibitors: NEM, which interacts with a critical sulfhydryl group on the catalytic subunit (28); DCCD, which binds covalently in the transmembrane proton channel (29); and bafilomycin A_1, a macrolide antibiotic isolated from *Streptomyces* sp. that appears to be a potent and specific inhibitor of vacuolar type H^+-ATPases (30, 31). As shown in Figure 4, inhibitory profiles and estimated 50% inhibitory concentrations were similar for EE and PG endosomes with NEM (13.5 and 18.1 μmol/L, respectively), DCCD (206 and 216 μmol/L, respectively) and bafilomycin A_1 (11 and 6 nmol/L, respectively). Thus EE treatment did not appear to alter the response to these inhibitors. We showed previously that acidification of EE endosomes is increased by cyclic AMP and protein kinase A to the same degree as control endosomes (33), suggesting that the response to second-messenger systems also is not affected by EE.

Although EE treatment reduced the initial ATP-dependent rates of endosome acidification, it had no discernible effect on steady-state intravesicular $[H_i^+]$, either before or after addition of MgATP (Fig. 5). In the presence of MgATP, steady-state $[H_i^+]$ increased as medium chloride increased, reflecting dissipation of $\Delta\Psi$ and an increase in ΔpH (4, 27, 32). However, EE treatment did not alter these values.

Because steady-state $[H_i^+]$ reflects the algebraic sum of proton influx and efflux, we also measured passive proton efflux rates in KCl and potassium gluconate medium; results are shown in Figure 6. Proton efflux rates reflect the combined effects of proton chemical gradients, membrane potential, intrinsic proton permeability and permeability and gradients of the counterions, principally K^+ and Cl^-. In these studies, proton gradients ($[H^+_o] - [H^+_i]$) were similar for EE and PG endosomes (1.2×10^{-6} mol/L H^+ [EE and PG] in KCl medium and 0.67×10^{-6} mol/L H^+ (EE) and 0.69×10^{-6} mol/L H^+ (PG) in potassium gluconate media).

For both EE and PG endosomes, proton efflux rates were faster in potassium gluconate medium than in KCl medium and this likely primarily reflects the considerable positive ATP-dependent $\Delta\Psi$ that develops in endosomes and other acidified vesicles in potassium gluconate medium (4, 23). Indeed, the addition of valinomycin to potassium gluconate medium, which would be expected to reduce the interior positive $\Delta\Psi$ (25, 32, 34) reduced proton efflux rates in both EE and PG endosomes.

By contrast, in KCl medium, the addition of valinomycin increased proton efflux rates in both EE and PG endosomes. Because in Cl^- medium $\Delta\Psi$ would be expected to be low (4), this observation suggests that in both EE and PG endosomes, proton permeability is greater than Cl^- permeability and the latter becomes rate-limiting, resulting in development of an interior negative $\Delta\Psi$ that slows proton efflux. Valinomycin, by

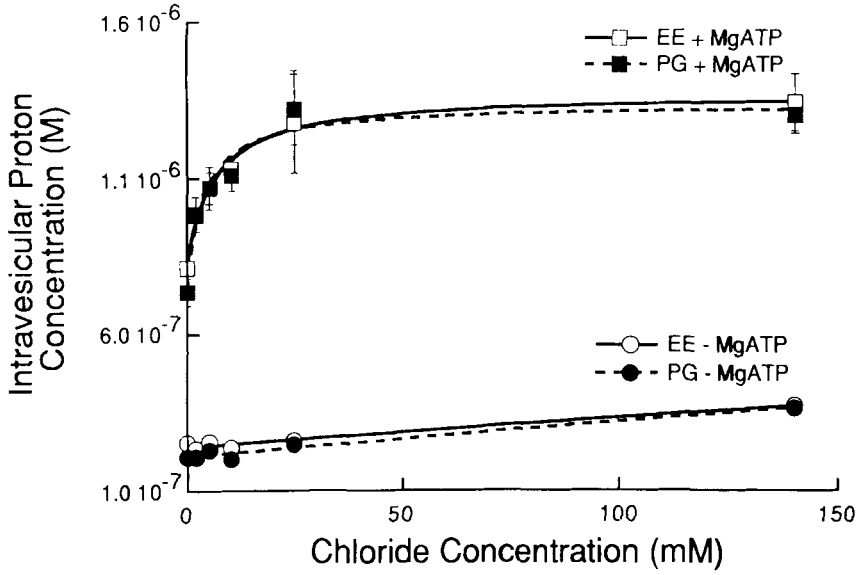

FIG. 5. Steady-state intravesicular proton concentration plotted against medium chloride concentration for EE-treated and PG-treated endosomes measured in the absence (○, ●) or presence (□, ■) of MgATP. Symbols represent the mean ± S.E.M. of values obtained from four to 14 different paired preparations of EE and PG endosomes. S.E.M. bars smaller than the size of symbols are not visible. Curves represent the best-fit functions of the forms $y = ax/(b + x) + c$ (with MgATP) and $y = ax + b$ (without MgATP).

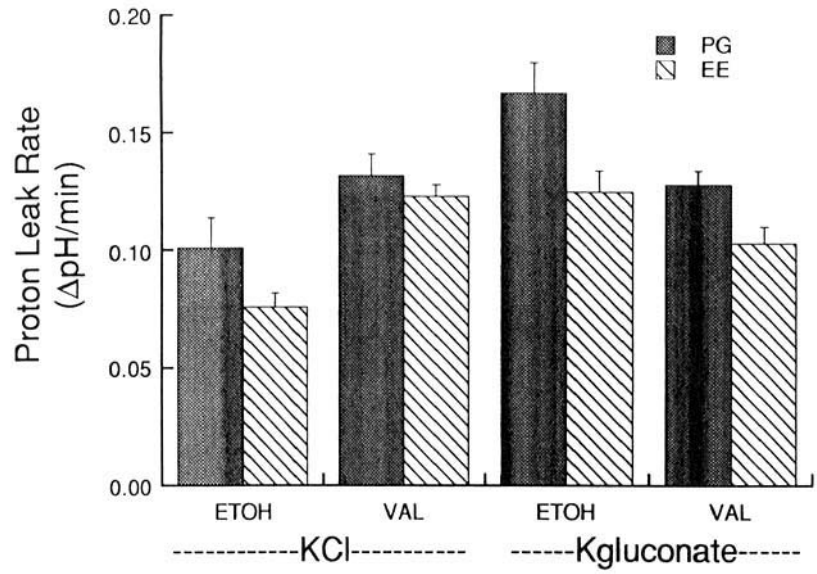

FIG. 6. Effect of EE on endosome proton leak rates. Endosomes treated with EE *(hatched bars)* and PG *(dark bars)* were prepared and resuspended in buffer containing 30 mmol/L Bis-Tris (pH 7.0) and 140 mmol/L KCl or 140 mmol/L potassium gluconate. Leak rates were measured, as described in Materials and Methods, after addition of small aliquots of ethanol *(ETOH)* or valinomycin *(VAL)*. Bars represent the mean ± S.E.M. of values from four or five separate paired preparations of EE-treated and PG-treated endosomes. Mean pH_i values when leak rates were measured were 5.87 (EE and PG) in KCl medium and 6.09 (PG) and 6.10 (EE) in potassium gluconate medium. Although we noted a trend toward slower proton leak rates in EE endosomes, none of these differences were statistically significant.

increasing K^+ permeability (P_{K+}) reduces the negative $\Delta\Psi$ and thus increases proton efflux.

Under all the conditions tested, EE endosomes exhibited slower proton efflux rates than PG endosomes, although none of these differences were statistically significant (Fig. 6). These observations suggest that EE treatment modestly reduced endosome proton permeability.

Using values for buffering capacity, we calculated proton efflux rates (millimoles per minute) for EE and PG endosomes in KCl medium with valinomycin, conditions likely to minimize any vesicle $\Delta\Psi$, and obtained values of 5.10 (EE) and 5.65 (PG) mmol/min. These values and the $([H^+_i] - [H^+_o])$ (1.2×10^{-6} mol/L H^+ for both EE and PG endosomes in KCl medium) were used to obtain estimates for membrane proton perme-

ability (P_{H^+}) of 4.25×10^3/min (EE) and 4.71×10^3/min (PG) with EE values 11% lower than PG values. These values are in the range we measured for P_{H^+} for rat liver endosomes (10.7×10^3/min), lysosomes (1.27×10^3/min) and multivesicular bodies (33.3×10^3/min) (22, 23; Van Dyke RW, Unpublished observations, 1990-1992). Thus EE treatment may modestly decrease initial rates of acidification and proton efflux rates, resulting in little discernible effect on steady-state, ATP-dependent pH_i.

Finally, the findings illustrated in Figure 6 suggest that valinomycin decreased the differences in proton efflux between EE and PG endosomes in KCl media. This observation might be explained if EE treatment affected not only proton but also K^+ permeabilities. If so, then in KCl medium, EE endosome proton efflux rates would be limited by both decreased P_{H^+} and P_{K+}, whereas in KCl medium with valinomycin, proton efflux would be limited (compared with PG endosomes), mainly by decreased P_{H^+}.

DISCUSSION

EE affects several liver functions. It has been repeatedly shown to cause cholestasis (14-17, 19, 35); to impair bile acid transport (17-19), Na,K-ATPase (13-17) and Ca^{+2}-ATPase (20); and to alter cytochrome P-450 (36). EE also appears to alter endocytic and vesicular traffic of a variety of proteins. It increases release of lysosomal enzymes and lysosomal contents into bile (37), decreases the transcytotic movement of IgA from blood to bile (12) and increases the proportion of ASOR subjected to endocytosis that escapes lysosomal degradation and is released intact in bile (12). The mechanisms by which EE exerts all of these effects are not well understood; however, possible mechanisms include changes in (a) membrane cholesterol ester and phospholipid composition (13, 15, 16); (b) membrane fluidity (13-16); (c) synthesis, function or both of proteins (17-19, 36); and (d) formation, sorting, targeting and fusion of endocytic and exocytic intracellular vesicles (12, 37).

Endocytic vesicles are acidified by an electrogenic proton pump or vacuolar H^+-ATPase that functions in conjunction with a halide conductance to maximally acidify the interior of vesicles while minimizing $\Delta\Psi$ (2-5, 32). Endocytic vesicles progressively acidify after formation, with lysosomes exhibiting the most acidic pH_i (8-10). Although the proton pump responsible for vesicle acidification has been identified in many intracellular organelles, little is known about potential regulatory mechanisms for this pump. Because EE has been shown to alter a number of steps in receptor-mediated endocytosis, we embarked on this study to determine whether this hormone alters endosome acidification. For these studies, we employed a conventional treatment protocol that reproducibly produces cholestasis in rats (13-16); each estrogen-treated rat was treated in parallel with a littermate control animal. Endosomes were prepared simultaneously from each of the paired animals (EE and PG) on each experimental day, and vesicles were studied under identical conditions. For this study, we chose to examine acidification in all endocytic vesicles formed over a 10-min period to include very early and sorting endosomes, such as compartment for uncoupling of receptors and ligands vesicles (CURL vesicles) in our experiments. Also, we employed a technique for assessing endosome acidification that is independent of the number of vesicles studied and vesicle FITC-dextran content. Thus our results would not be affected by differences in the number or size of endocytic vesicles formed during exposure to FITC-dextran (21-24).

Whether the endosomes prepared from EE-treated rats are the same vesicles prepared from PG-treated rats cannot be definitively answered from our studies; techniques to unambiguously define and isolate subsets of early endocytic vesicles are not well developed. In addition, endosome populations may be defined by one or more of several sets of criteria, including morphological shape, content of receptors and ligands, time elapsed since formation, composition of membrane proteins, derivation from coated-pit or non-coated-pit areas and presence of various proteins that determine endosome trafficking. After formation, endosomes rapidly remodel and sort contents into a variety of vesicles destined for many different pathways. Thus criteria are likely to define overlapping rather than distinct populations of vesicles. We therefore chose to study vesicles loaded with the fluid-phase marker FITC-dextran to study the effects of EE on ion transport of all endocytic vesicles formed by hepatocytes in a defined time period.

The principal finding of this study was that treatment with EE resulted in small but consistent and statistically significant decreases in the initial rates of acidification in FITC-dextran–loaded endocytic vesicles. The mechanism of this change was not established from our studies; however, ion-substitution studies (Table 2, Fig. 2), inhibitor profiles (Fig. 4) and MgATP kinetics (Fig. 3) suggest that EE did not alter the subunit structure of the proton pump, its affinity for ATP, rate limitations imposed by chloride availability or general responses to ion substitution. Our findings indicate that the V_{max} of acidification was decreased (Fig. 2), an observation usually attributed to a decrease in the number or turnover rate of pump units.

These differences in acidification between EE-treated and PG-treated endosomes may represent changes in one or more of the following factors: (a) number of proton pumps inserted per unit of endosome membrane surface area during vesicle formation or during endosome remodeling, (b) vesicle surface-to-volume ratio (22), (c) proton pump turnover rate, (d) vesicle buffering capacity, (e) counterion permeability, (f) presence of other electrogenic ion pumps (38) and (g) regulatory factors such as phosphorylation by protein kinase A (33). EE treatment could affect any one or more of these factors, some of which could be considered as defining a different "subset" of endosomes. For example, EE might alter synthesis or correct insertion of proton pumps into endocytic vesicles, as has been proposed to

explain the effects of estrogen on cytochrome P-450 (36) and the canalicular ATP-dependent bile acid transporter (19). Alternatively, because we have shown that early endosomes (prepared after 2-min or 10-min exposure to FITC-dextran) acidify faster than more "mature," later endosomes (CURL vesicles and multivesicular bodies), which in turn acidify faster than lysosomes (22; Van Dyke RW, Unpublished observations, 1990-1992), it is possible that EE modestly changes the rates of formation or remodeling and maturation of endosomes such that vesicles formed over 10 min by EE-treated livers included more "mature" vesicles with decreased rates of acidification.

EE also might alter the turnover rate of individual proton pumps, possibly through changes in endosome lipid composition. The vacuolar H^+-ATPase activity isolated from brain clathrin-coated vesicles has been shown to be sensitive to changes in membrane phospholipid and cholesterol composition (39), and EE is known to increase cholesterol esters, sphingomyelin and several phospholipids in hepatic plasma membranes (15, 16). It is not known, however, whether estrogen alters the lipid composition of endocytic vesicles (lipid composition of our endosomes could not be assessed because the preparation was not pure) or whether changes in lipid composition alter proton transport, as opposed to ATP hydrolysis, of the vacuolar proton pump.

Our results also suggest that EE treatment alters endosome K^+ permeability, as indicated by the smaller effects of EE on acidification in $NMDG^+$ medium compared with that in K^+ medium and by the lesser effects of EE on proton leak rates in KCl medium with valinomycin. Further studies that directly assess K^+ permeability and determine its role in regulating endosome acidification will be needed to confirm this possibility.

Finally, our own studies suggest that the last two factors are unlikely explanations for the effects of EE. We have shown similar effects of cAMP and protein kinase A on EE and control endosomes (33), and we have failed to identify functionally significant Na,K-ATPase in rat liver early endosomes (38).

Clearly, additional studies using highly purified and well-characterized populations of endosomes or of purified proton pumps will be required to differentiate effects of estrogens on pump turnover rate from effects on membrane proteins and on lipid composition or on the "maturity" or geometric configuration of endosomes.

EE not only affected initial rates of endosome acidification; it also reduced vesicle proton leak rates, measured either as ΔpH/min (Fig. 6) or as a flux (in mmol/min). Indeed, these two changes appeared to counterbalance each other to the extent that estrogen did not alter steady-state ATP-dependent pH_i (Fig. 5). To examine this issue further, we calculated apparent proton permeability coefficients from flux rates and the transmembrane proton gradient (25) under conditions (KCl medium with valinomycin) that would be expected to minimize $\Delta\Psi$. Biological membranes exhibit a relatively high passive permeability for protons compared with other ions (40), and the values we measured in this study were similar to values measured in a variety of epithelial plasma membranes (25, 34, 41, 42). EE treatment decreased P_{H^+} by about 11%. The mechanism for these changes in P_{H^+} is not known, but because increases in plasma membrane fluidity have been correlated to increases in P_{H^+} in renal and gastroduodenal apical plasma membranes (41, 42), it is possible that estrogen-induced decreases in endosome P_{H^+} are also related to decreased membrane fluidity. These changes in P_{H^+} are not likely due to the effects of increased bile salts in cholestatic liver; bile salts increase rather than decrease P_{H^+} in duodenal membrane vesicles (43).

Overall, our findings indicate that after treatment with EE, rat liver endosomes formed over a 10-min period exhibit modestly decreased rates of acidification. These changes are small, but they might affect the efficiency of uncoupling of receptor from ligand during the early stages of receptor-mediated endocytosis because this step is dependent on modest changes in endosome pH_i. Because steady-state endosome pH_i was not affected by estrogen treatment, it is unlikely that estrogen affects acidification and function of late endocytic vesicles, such as lysosomes, that have presumably achieved steady-state pH_i. Our findings and those of others may provide a partial explanation for some of the EE-induced abnormalities in endocytosis noted by Goldsmith et al. (12). For example, chloroquine is known to impair endosome acidification and to partially alkalinize endocytic vesicles and lysosomes in intact cells (44, 45). Chloroquine, like estrogen, also delays biliary excretion of IgA and transfer of ligands subjected to endocytosis to lysosomes (46), suggesting that some of these abnormalities in handling ligands subjected to endocytosis may in part be related to small changes in vesicle acidification. Further studies correlating changes in vesicle pH_i with handling of macromolecules subjected to endocytosis in intact cells will be needed to confirm this hypothesis. Finally, it is possible that estrogens also affect other aspects of endocytosis such as vesicle formation, receptor sorting and membrane fusion.

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