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# CFTR DOES NOT ALTER ACIDIFICATION OF L CELL ENDOSOMES

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SUMMARY: Endosomes from L cells, transduced with the CFTR gene, and the parental line, which does not express detectable levels of CFTR, were loaded with FITC-dextran, isolated and the initial rates of acidification, steady-state $pH_i$, and proton leak rates were compared over a range of chloride concentrations (0-140 mM). Values for these parameters were similar for endosomes from both cell lines in the presence and absence of cAMP and PKA. These results indicate that CFTR does not alter L cell endosome acidification, possibly due to an adequate intrinsic $Cl^-$ conductance or to a failure to incorporate sufficient functional CFTR or a necessary co-factor in endocytic membranes.


An endosome acid interior pH ($pH_i$), important for receptor-mediated endocytosis and other vesicular functions, is mediated by an electrogenic $H^+$-ATPase and facilitated by a parallel $Cl^-$ conductance, which dissipates electrical gradients and allows an increase in proton chemical gradients (1,2). The cystic fibrosis transmembrane conductance regulator (CFTR) , a cAMP regulated $Cl^-$ channel (3), has been suggested to regulate acidification in endocytic vesicles (4) since an increase in endosome $Cl^-$ conductance might increase ATP-dependent vesicle acidification in $Cl^-$-containing media.

Abbreviations: CFTR, cystic fibrosis transmembrane conductance regulator; IBMX, 3-isobutyl-methylxanthine; PKA, protein kinase A; SPQ, 6-methoxy-N-(sulfopropyl)quinolinium; CCCP, carbonyl cyanide m-chlorophenylhydrazone; HEPES, N- [2-hydroxyethyl] piperazine-N'- [2-ethanesulfonic acid]; 8-CPT-cAMP, 8-(4-chlorophenylthio)-adenosine-3',5'-cyclic monophosphate; $F_{max}$, maximum fluorescence; F, fluorescence; FITC-dextran, fluorescein isothiocyanate-dextran; Bis-Tris, bis [2-Hydroxyethyl] imino-tris [hydroxymethyl] methane; $pH_i$, vesicle interior pH; $V_{max}$, maximum rate of acidification; $ED_{50}$, the concentration of substrate at half-maximal velocity.

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We examined acidification of endocytic vesicles from L cells transduced with CFTR (termed "C" cells) which demonstrate a forskolin-sensitive plasma membrane $Cl^-$ conductance. As controls, we also studied the parental line of L cells ("P" cells) and mock transduced cells ("M" cells).

## METHODS

Cell Culture: L cells were multiply transduced with the normal CFTR gene as previously described and exhibited about one copy per L cell genome (5). Western blots showed CFTR protein in C but not P cells (5). Mock transfected cells were serially exposed to polybrene without transfecting virus, cultured and cryopreserved as for transduced C cells. Immunofluorescent studies showed CFTR on the plasma membrane and some intracellular vesicles in C but not P or M cells (5). All cell lines were grown until confluent in modified Eagle' medium with 10% fetal bovine serum, 1% penicillin-streptomycin at 37°C with 5% $CO_2$ on 10 cm plastic dishes. SPQ Analysis of $I^-$ Flux: All cell lines were examined for cAMP-induced increases in $I^-$ efflux using the fluorescent $Cl^-$-sensitive dye SPQ as previously described (5,6) using a Zeiss epifluorescent microscope and Attofluor digital fluorescence imaging system. Cells were perfused, in sequence, for two minutes each with isotonic NaI buffer, $NaNO_3$ buffer, NaI buffer, and then the same buffers in the same sequence except containing 100μM 8-CPT-cAMP. Maximum fluorescence ($F_{max}$) was measured in $KNO_3$ buffer with 10μM nigericin and 10μM tri-n-butyltin cyanide. Fluorescence (F) of individual cells (13-41 from each coverslip) was expressed as $F_{max}/F$ (6). Slopes of the rate of change in SPQ fluorescence after switching from $I^-$ to $NO_3^-$ media were taken as a measure of $I^-$ efflux. Rates in the presence and absence of cAMP were expressed as a ratio. Ratios measured in CFTR-transduced C cells were considered to be increased if they were greater than the mean + 2 S.D. of the ratios measured in P and M cells. Isolated Vesicle Preparation: L cell early to late endosomes were loaded by incubating dishes of confluent cells with 25mg/ml fluorescein isothiocyanate-dextran (FITC-dextran) in buffer (130mM NaCl, 1mM Mggluconate, 1mM Cagluconate, 20mM HEPES, 4mM Kgluconate, 1mM $Na_2HPO_4$, 10mM glucose, pH 7.35) at 37°C for 30 minutes. Dishes were chilled and rinsed with a $Cl^-$-free version of this buffer, cells were scraped, homogenized and centrifuged (7). Pellets that contained endosomes were resuspended in Kgluconate buffer (140mM Kgluconate, 10mM Bis-Tris, pH 7.0) and kept on ice until used. For cAMP treatment, cells were exposed to 500μM dibutyryl cAMP, 10μM forskolin, and 500μM IBMX during the last 4 minutes of incubation with FITC-dextran. L cell lysosomes were prepared similarly except that cells were incubated with 2.5 mg/ml FITC-dextran for 18 hours at 37°C, and chased for 2 hours without FITC-dextran before cells were chilled and homogenized. Fluorescent Assays: ATP-dependent initial rates of acidification, steady-state $pH_i$ and proton leak rates were determined from the fluorescence of internalized FITC-dextran as described (8,9) in Bis-Tris buffers containing 140 mM $K^+$, 0, 2, 5, 10, 25, or 140 mM $Cl^-$ and sufficient gluconate to maintain tonicity. In some studies valinomycin (2.5μM) was added before measuring acidification or during proton leaks. For PKA treatment, endosomes were pre-incubated in buffer for 5 minutes at 22°C with 10μM MgATP and 25 units of the catalytic subunit of PKA (or solvent for control studies) before acidification was initiated by 5mM MgATP. Findings in P and M cells were identical and combined. Plots of initial rates of acidification versus medium concentration of $Cl^-$ were fit to a function of the form $y = ax/(b+x) + c$ ($a+c=V_{max}$, $b=ED_{50}$) as described (8,9). Statistics: Results were expressed as mean ± SEM ($n \geq 3$) of assays in "n" different cells or preparations of vesicles and differences were analyzed with t tests. Significance was taken as $p<0.05$.

## RESULTS

C cells expressed cAMP-dependent halide transport as assessed by SPQ fluorescence changes. The ratio of rates of $I^-$ efflux (with cAMP/without cAMP) in P and

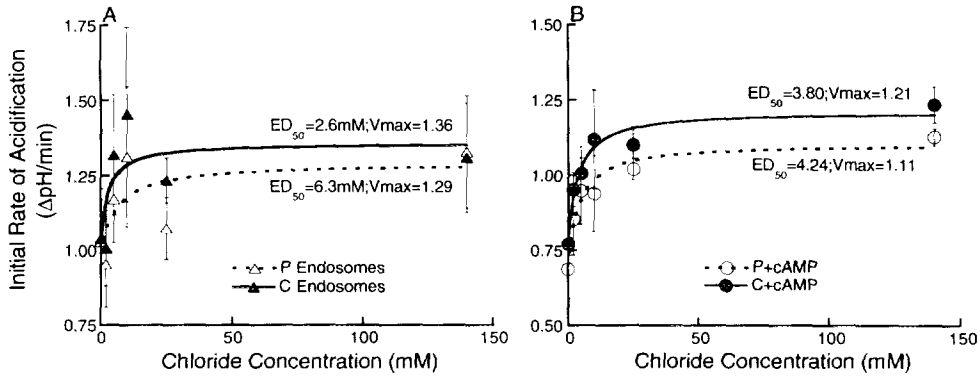


Figure 1. Initial rates of acidification of P (open symbols) and C (closed symbols) endosomes in media with 0 to 140mM $Cl^-$. Initial rates are expressed as mean ± SEM. Estimates for the parameters $ED_{50}$ and $V_{max}$ of fitted curves are indicated on the graphs. (A) Endosomes prepared from untreated P and C cells (n=5-11). (B) Endosomes prepared from P and C cells exposed to dibutyryl cAMP, forskolin and IBMX (n=3).

M cells was 0.99 ± 0.04 (n=13) and the upper limit of control ratios (mean + 2 S.D.) was 1.27. In three separate experiments, ratios of rates of $I^-$ efflux in C cells averaged 1.56 ± 0.10 (n=24), 1.75 ± 0.08 (n=41) and 1.89 ± 0.12 (n=34) and 69%, 93% and 85%, respectively, of individual C cells exhibited increased ratios of cAMP-induced $I^-$ efflux.

Initial rates of endosome acidification are shown in Figure 1A. There was no significant difference between acidification rates in endosomes from the parental cells (P) and CFTR-transfected cells (C) either in the presence or absence of $Cl^-$, although values tended to be slightly higher in C endosomes. Kinetic analysis gave similar estimates for maximum rate ($V_{max}$) of 1.3 and 1.4 ΔpH/min and $ED_{50}$ values for $Cl^-$ of 6.3 and 2.6 mM in P and C endosomes, respectively. Steady-state ATP-dependent $pH_i$'s (5.78 and 5.72 in 140 mM $Cl^-$ for P and C cells, respectively) also were not different at any $Cl^-$ concentration (data not shown).

If CFTR or other PKA-responsive $Cl^-$ conductances were present in C endosomes, opening these conductances by cAMP or PKA would be expected to increase both initial rates of acidification and proton leak rates when proton fluxes are rate-limited by movement of counter-ions. Exposure of L cells to dibutyryl cAMP, forskolin and IBMX for four minutes had no significant effect on acidification rates of subsequently prepared endosomes (Figure 1B), or steady-state $pH_i$ (data not shown), whether vesicles were prepared from parental or CFTR-transduced cells, although there was a trend towards decreased rates of acidification in cAMP-treated vesicles from both cell lines. Compared to rates of acidification in Kgluconate medium, 140mM $Cl^-$ increased rates by 26-29% in untreated P and C endosomes and by 60-64% in cAMP-exposed P and C endosomes, suggesting that cAMP may alter the relative response to $Cl^-$ in both cell lines, possibly via an endogenous cAMP-sensitive $Cl^-$ conductance.

We also examined acidification of endosomes treated with PKA *in vitro* for 5 minutes. PKA treatment increased rates of acidification in 2mM $Cl^-$ ($p<0.001$) and 25

**Table I. Rates of Acidification and Proton Leaks from Endosomes and Lysosomes**

| Vesicle | 2mM Cl- Buffer | | 25 mM Cl- Buffer | | |
|---|---|---|---|---|---|
| | Rate of Acidification (ΔpH/min) | Proton Leak Rate (ΔpH/min) | Rate of Acidification (ΔpH/min) | Proton Leak Rate (ΔpH/min) | Proton Leak Rate + valinomycin (ΔpH/min) |
| P Endosomes | | | | | |
| Control | 0.72±0.05 | 0.13±0.01 | 1.02±0.07 | 0.12±0.01 | 0.16±0.02 |
| +PKA | 0.95±0.05$^{+}$ | 0.13±0.01 | 1.20±0.12 | 0.12±0.01 | 0.15±0.01 |
| C Endosomes | | | | | |
| Control | 0.83±0.06 | 0.12±0.02 | 1.11±0.07 | 0.11±0.01 | 0.16±0.03 |
| +PKA | 0.95±0.06$^{+}$ | 0.10±0.01 | 1.26±0.12 | 0.10±0.01 | 0.13±0.01 |
| P Lysosomes | 1.42±0.21 | 0.06 | 1.19±0.19 | 0.05±0.01$^{***}$ | 0.06±0.01$^{**}$ |
| C Lysosomes | 1.32±0.12 | 0.07 | 1.54±0.34 | 0.07±0.01$^{*}$ | 0.08±0.01 |

n=4 for endosomes and 2-3 for lysosomes; $^{*}$ $p<0.05$ vs endosomes; $^{**}$ $p<0.01$ vs endosomes; $^{***}$ $p<0.001$ vs endosomes; $^{+}$ $p<0.02$ vs respective control.

mM $Cl^-$ (p=NS) media for both P and C endosomes (Table I). The lack of a specific effect of cAMP and PKA was not related to lack of rate limitation by counter-ion flux as, in 25mM $Cl^-$ medium, pre-incubation with 2.5μM valinomycin, which also neutralizes the change of protons by facilitating $K^+$ flux, increased rates of acidification by 33 and 37%, respectively in both P and C endosomes (data not shown).

*Proton leak rates, measured from fully acidified endosomes as the initial rate of* realkalinization after the proton pump was abruptly stopped by 1μM bafilomycin (8,9), did not differ in P and C endosomes either without or with exposure to PKA (Table I). Valinomycin consistently increased proton leak rates in 25mM $Cl^-$ media by 33 and 45% in control P and C endosomes (p=NS), respectively and by 26% in PKA-exposed P (p=NS) and C ($p<0.05$) endosomes (Table I).

Since we have shown that lysosomes from several other cell types do not appear to contain CFTR and exhibit only a limited $Cl^-$ conductance (7,9), we performed studies to confirm that the endosome population we employed differed from lysosomes. Rates of acidification of lysosomes measured in 0-140 mM $Cl^-$ were similar in P and C cells (Table I). Estimates for $V_{max}$ were 1.5 and 1.6 ΔpH/min, and for $ED_{50}$ were 1.1 and 3.8mM, for P and C lysosomes, respectively. These lysosomes could be readily distinguished from the endosome preparation by two features. First, steady-state ATP-dependent $pH_i$ was significantly more acidic in both P and C lysosomes (5.01 in 140mM $Cl^-$) compared to P and C endosomes (5.78, 5.72 in 140mM $Cl^-$) ($p<0.001$) at all six $Cl^-$ concentrations tested (data not shown). Second, proton leak rates were consistently lower in P and C lysosomes than in endosomes ($p<0.05$ in 25mM $Cl^-$) (Table I). These lower proton leak rates may account, in part, for the more acid steady-state $pH_i$ in lysosomes.

To test whether $Cl^-$ flux was rate-limiting for proton efflux, we examined proton leak rates after addition of bafilomycin and CCCP to endosomes acidified in 25mM $Cl^-$

medium without or with PKA treatment. In all studies, proton leak rates with CCCP were fast and difficult to measure accurately, indicating that counter-ion fluxes were not rate-limiting. Rates in P and C endosomes (2.08±0.41, 2.95±0.83 ΔpH/min without PKA and 2.27±0.36, 3.05±0.90 ΔpH/min with PKA, respectively) did not differ (p=NS).

## DISCUSSION

As regulation of $Cl^-$ conductance might affect acidification rates, steady-state $pH_i$ and proton leak rates of endosomes and lysosomes, we undertook this study to determine whether successful transduction of an L cell line, that does not normally express CFTR, with the normal CFTR gene would alter endosome acidification. The transduced cells we employed for these studies expressed CFTR message and protein (5), showed immunofluorescent localization of CFTR to the plasma membrane and to at least some intracellular vesicles (5) and >80% of cells exhibited a cAMP-dependent increase in plasma membrane halide conductance (5 and this study). Thus, many of these cells correctly synthesized and processed the CFTR protein and inserted it into the plasma membrane. We studied a mixed population of early to late endosomes, (that could be distinguished from lysosomes) to avoid missing effects of CFTR on only a subset of endosomes.

Endosomes from the nontransduced P cell line exhibited ATP-dependent acidification that was increased by 140mM $Cl^-$ (~29%) or by valinomycin (~35%), thus parental L cell endosome acidification appeared to be rate limited by charge compensating ions and CFTR, if inserted into endosome membranes, might be expected to increase endosome acidification rates. CFTR also would be expected to increase proton leak rates from endosomes acidified in high $Cl^-$ media as leak rates were increased by valinomycin. By contrast, acidification of endosomes from rat liver is increased >100% by similar changes in buffer $Cl^-$ (9), indicating considerably greater endogenous $Cl^-$ conductance in hepatic endosomes.

C endosomes, prepared from transduced L cells, acidified similarly to P endosomes in the presence and absence of $Cl^-$ without and with treatment with cAMP or PKA. Although C endosomes tended to acidify faster than P endosomes, these differences were not significant and are unlikely to be related to CFTR as they were observed in the absence of $Cl^-$. Although cAMP exposure tended to decrease and PKA treatment to increase endosome acidification, these effects were small, not statistically significant and equal for both P and C endosomes and thus unrelated to CFTR.

Steady-state $pH_i$ and proton leak rates also did not differ between P and C endosomes in either low (2mM) or high (25mM) $Cl^-$ or in the absence or presence of valinomycin. Indeed, studies with CCCP showed that proton leak rates were not predominantly limited by counter-ion movement.

Therefore, although intrinsic $Cl^-$ permeability of L cell endosomes appears to be, if anything, less than in rat hepatic endosomes, successful transduction with CFTR did not alter any parameters of endosome (or lysosome) acidification. Explanations for this lack of effect might include little or no sorting of functional CFTR to endosomes or lack of an

essential cofactor. Our findings are consistent with other investigators who observed that cAMP or forskolin did not increase initial acidification rates in endosomes from CFTR-transfected CHO or 3T3 cells (10-12). However these other groups have noted that proton leak rates, measured in endosomes in intact CFTR-overexpressing CHO, 3T3 or T84 cells after exposure to CCCP, were increased by 140-190% by forskolin in high $Cl^-$ media (10,12). Explanations for the differences in our findings and those of others include: 1) our cell line does not heavily overexpress CFTR (possibly reflecting a more physiologic distribution of CFTR protein; 2) our endosome preparation probably includes more mid-to late-stage endosomes that might be more likely to lack CFTR (12,13); and 3) L cell endosome proton efflux appears to be more rate-limited by proton permeability and less affected by counter-ion flux (10-12). Thus the effect of CFTR on acidification of intracellular organelles may exhibit considerable cell and species variability and also may depend on the other ion transporters present in the specific organelle under study.

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