Acute Mechanism of Medium Chain Fatty Acid-Induced Enhancement of Airway Epithelial Permeability

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ABSTRACT
The localization of viral receptors to the basolateral surface of airway epithelia is an obstacle to the effectiveness of luminal viral-mediated gene transfer to the lung. The tight junction (TJ) serves as a rate-limiting barrier to the penetration of viral vectors. We have previously identified the sodium salt of the medium chain fatty acid (MCFA) capric acid (C10) as an agent that can enhance the ability of adenoviral vectors to transduce well differentiated (WD) primary human airway epithelial (HAE) cells. Previous studies have suggested that intracellular calcium (Ca$^{2+}$) levels may play a central role in the long-term C10-mediated increases in junctional permeability. In this study, we investigated the effects of C10 and lauric acid (C12) on Ca$^{2+}$ in WD primary HAE cells and determined whether these effects were necessary for the acute MCFA-induced reduction in transepithelial resistance (R(T)) and increased permeability. In addition, we characterized the effects of C10 and C12 on components localized to the TJ, including ZO-1, junctional adhesion molecule (JAM), and the claudin family of transmembrane proteins. In addition to rapidly decreasing R(T), C10 and C12 increased cellular and paracellular permeability. C10 induced a rapid, sustained increase in Ca$^{2+}$. However, buffering Ca$^{2+}$ did not block the effects of C10 on R(T). Both C10 and C12 caused reorganization of claudins-1, -4, JAM, and β-catenin, but not ZO-1. These data suggest that C10 and C12 exert their acute effects on airway TJs via a Ca$^{2+}$-independent mechanism of action and may alter junctional permeability via direct effects on the claudin family of TJ proteins.

Tight junctions (TJs) play a central role in sealing the intercellular space between the apical and basolateral compartments of epithelial and endothelial cells. Modulation of the barrier function of the TJ has been investigated as a strategy to enhance transmucosal drug absorption, particularly to increase the bioavailability of drugs after rectal administration (Morimoto et al., 1989; Yamazaki et al., 1990; Anderberg et al., 1993; Kinouchi and Yata, 1996; Yamamoto et al., 1996; Shimazaki et al., 1998; Soderholm et al., 1998). The sodium salts of several medium chain fatty acids (MCFAs), particularly capric (C10) and lauric (C12) acids, have been shown to increase rectal drug absorption, presumably by causing alterations in intestinal TJ barrier function. C10 has also been shown to lead to profound alterations in the barrier function of the airway TJ and has been investigated as an agent to enhance viral-mediated airway gene transfer (Coyne et al., 2000). These agents are attractive as potential therapies to enhance absorption of gene transfer vectors due to the rapid onset of action (within minutes) and their relatively rapid recovery (within hours) after treatment (Lindmark et al., 1995, 1998; Tomita et al., 1995, 1996; Coyne et al., 2000).

Although MCFA’s have been widely investigated as agents to increase the delivery of therapeutic agents, relatively little is known regarding their primary mechanism of action. The ability of intercellular TJs to function as a barrier to the diffusion of macromolecules is dynamically regulated by numerous intracellular signals and the permeability properties of the TJ vary in response to changes in physiological state. Therefore, agents that regulate the TJ likely do so by direct or indirect actions on intracellular signals and/or the protein components of the TJ.

The integrity of the intercellular junctions requires a finite

ABBREVIATIONS: TJ, tight junction; MCFA, medium chain fatty acid; C10, capric acid; C12, lauric acid; Ca$^{2+}$, intracellular calcium; PLC, phospholipase C; HAE, human airway epithelial; R(T), transepithelial resistance; JAM, junctional adhesion molecule; BAPTA-AM, 1,2-bis(2aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid-acetomethyl ester; Ad, adenovirus; PBS, phosphate-buffered saline; HBR, HEPES-buffered Ringer; TR, Texas Red; C8, caprylic acid; βgal, β-galactosidase; CAR, coxsackie virus B and Ad2/5 receptor; ER, endoplasmic reticulum; U73122, 1-[6-[(17β)-3-methoxyestr-1,3,5(10)-trien-17-yl]amino]hexyl-1H-pyrole-2,5-dione; 48/80, condensation product of N-methyl-p-methoxyphenyl-ethylamine with formaldehyde; W7, 1-[(5-chloronoraphthalene-1-sulfonfonyl)-1H-hexahydropyridine-1,4-diazepine hydrochloride; KN62, (S)-5-isoquinolinesulfonic acid; H7, 1-(5-isoquinolinesulfonfonyl)-2-methylpipеразин; AM, acetoxymethyl ester.
concentration of extracellular Ca\(^{2+}\). Removal of Ca\(^{2+}\) by chelators such as EGTA leads to significant increases in paracellular permeability and gene transfer efficiency (Duan et al., 1998; Wang et al., 1998; Coyne et al., 2000). The role of intracellular Ca\(^{2+}\) (Ca\(^{2+}\)) in TJ regulation has also been well studied. Studies in Caco-2 cells have suggested that the increase in C10-induced permeability may be a result of the elevation of Ca\(^{2+}\) via a phospholipase C (PLC)-dependent pathway (Tomita et al., 1995, 1996; Lindmark et al., 1997, 1998). Pharmacological inhibition of PLC, myosin light chain kinase, and calcium calmodulin have been shown to reduce the C10- and C12-induced alterations in TJ integrity in Caco-2 cells (Lindmark et al., 1998).

These inhibition studies, implicating a role for PLC in the effect of C10 on permeability, focused on the relatively long-term effect of exposure (12–60 min) (Lindmark et al., 1998). However, the effects of C10 on paracellular permeability occurred within seconds of exposure in both Caco-2 and WD primary HAE cells (Tomita et al., 1995; Coyne et al., 2000), and inhibition of PLC in Caco-2 cells did not prevent the acute increase in permeability. The role that the increase in Ca\(^{2+}\) levels plays in the acute mechanism of C10 and C12, which have the similar acute effects on the TJ, remains unclear but both agents likely mediate their initial effects via a similar path (Lindmark et al., 1995). Because these compounds remain attractive as enhancers of therapeutic drug or vector absorption, a clearer understanding of the acute mechanism of action of these compounds is essential.

To determine the acute mechanism of action of C10 and C12 in the airway epithelium, we incubated primary human airway epithelial (HAE) cells with C10 and C12 and determined the effects of this treatment on transepithelial resistance (R\(_{te}\)), paracellular permeability, and luminal gene transfer efficiency. We then examined the effects of MCFA exposure on the level of Ca\(^{2+}\) and determined whether buffering Ca\(^{2+}\) to high and low levels affected the C10-induced changes in permeability. We then characterized the effects of C10 and C12 on structural components of the intercellular junction, including JAM, claudins-1 and −4, actin, and β-catenin.

**Materials and Methods**

**Chemicals and Antibodies.** The sodium salts of C10 and C12 were purchased from Sigma-Aldrich (St. Louis, MO). Rabbit polyclonal antibodies to ZO-1, claudin-1, and β-catenin and mouse monoclonal antibody to claudin-4 were purchased from Sigma-Aldrich (St. Louis, MO). The sodium salts of C10 and C12 were used because they have the similar acute effects on the TJ, remains unclear but both agents likely mediate their initial effects via a similar path (Lindmark et al., 1995). Because these compounds remain attractive as enhancers of therapeutic drug or vector absorption, a clearer understanding of the acute mechanism of action of these compounds is essential.

**Cell Culture.** Primary airway cells from human subjects were purchased from Sigma-Aldrich. The sodium salts of C10 and C12 were used because they have the similar acute effects on the TJ, remains unclear but both agents likely mediate their initial effects via a similar path (Lindmark et al., 1995). Because these compounds remain attractive as enhancers of therapeutic drug or vector absorption, a clearer understanding of the acute mechanism of action of these compounds is essential.

**Transient Plateau Cell Permeability.** To determine changes in Ca\(^{2+}\) with confocal microscopy, cultures were then mounted on a glass coverslip over an objective coupled to a confocal microscope. XZ-scans were recorded at the indicated time points.

**Measurements of Ca\(^{2+}\).** Primary HAE cells were treated with 150 μl of vehicle, C10, or C12 in nominally Ca\(^{2+}\)-free HEPES-buffered Ringer (HBR) (130 mM NaCl, 5 mM KCl, 1.3 mM MgCl\(_2\), 10 mM HEPES pH 7.4, 1.3 mM CaCl\(_2\), and 5 mM glucose) containing 2 mg/ml Texas Red (TR)-labeled dextran of 30 and 200 kDa while mounted on a glass coverslip over an objective coupled to a confocal microscope. XZ-scans were recorded at the indicated time points.

**ATP Depletion.** ATP depletion was accomplished by inhibition of both oxidative and glycolytic pathways. Briefly, glycolysis was inhibited by 2-h incubation at 37°C of primary HAE cells in a modified glucose-free Ringer's solution containing 2 mM glutamate in the apical and basolateral compartments as described previously (Bacalao et al., 1994). After this incubation, ATP was rapidly depleted by bathing cells apically in 10 μM antimycin A and 2-deoxyglucose (10 μM) (Bacalao et al., 1994), and R\(_{te}\) was determined at indicated time points.

**Immunofluorescent Labeling and Confocal Microscopy.** Cells were permeabilized with methanol at −20°C for 30 min. Antibodies to ZO-1, claudin-1, β-catenin, and claudin-4, and DNM diluted to 1:1000 were added to the luminal surface for 1 h. Cells were washed with PBS, and TR-labeled secondary antibodies (Amersham...
Biosciences Inc., Piscataway, NJ) diluted 1:600 in 10% goat serum/PBS were added to the luminal surface. For JAM and ZO-1 double labeling, 3D8 mouse anti-JAM and rabbit anti-ZO-1 were added to the cultures. After washing, anti-rabbit TR and anti-mouse fluorescein isothiocyanate antibodies were incubated in 10% goat serum/PBS for 1 h at room temperature. For actin staining, cultures were permeabilized in 1% Triton X-100, washed three times with PBS, and incubated with 1:250 dilution of Oregon Green phalloidin (Molecular Probes) for 30 min at room temperature and washed in PBS. Cells were postfixed with 4% paraformaldehyde. Transwell-Col inserts were excised and mounted on slides with 100 μl of Vectashield (Vector laboratories, Burlingame, CA) containing 4,6-diamidino-2-phenylindole. Images were captured with a confocal laser scanning microscope (Leica, Exton, PA).

**Statistics.** Data are presented as mean ± S.E.M. A one-way analysis of variance and Bonferroni’s correction for multiple comparisons were used to determine statistical significance (p < 0.05).

**Results**

**MCFA-Induced Alterations in TJ Barrier Function.** To evaluate the acute effects of MCFAs on permeability, we measured the effect of 30 mM caprylic (C8), 30 mM capric (C10), and 10 mM lauric (C12) treatment on **R**<sub>T</sub> at 0, 15, 30, 60, and 300 s (Fig. 1A). By 15 s post-treatment, **R**<sub>T</sub> in cultures exposed to C10 and C12 already showed a pronounced decrease in **R**<sub>T</sub>, corresponding to **R**<sub>T</sub> values of 46.9 ± 5.5% (C10) and 32.7 ± 3.1% (C12) of vehicle-treated controls. By 60 s, **R**<sub>T</sub> had decreased to 19.4 ± 2.3% (C10) and 14.3 ± 1.5% (C12) of control levels. However, C8 has no effect on **R**<sub>T</sub> at any time point. Upon removal of C10 and C12, **R**<sub>T</sub> increased over 12 h, and returned to vehicle-treated levels by 24 h post-treatment (Fig. 1B).

The effects of C8, C10, and C12 on **R**<sub>T</sub> and gene transfer efficiency were compared after treatment of primary HAE cells for 2 to 5 min. Optimal concentrations for each MCFA, based on kinetics of effect and recovery, were determined to be 30 mM C10 and 10 mM C12 (data not shown). Cells were treated with C10 and C12 until a maximal fall in **R**<sub>T</sub> values occurred with levels 5% of vehicle-treated controls. C8 caused a negligible change in **R**<sub>T</sub> (95% of vehicle controls) over this same treatment period and its effects were negligible even at doses of 100 mM and incubation times up to 60 min (data not shown). Therefore, C10 and C12 were identified as having significant effects on airway barrier function.

Subsequently, we infected primary HAE cells pretreated with C8, C10, or C12 and evaluated the effect on transduction efficiency of AdlacZ 48 h later. Transduction efficiency was low in cultures treated with vehicle alone or C8 (1.55 ± 0.036 or 3.57 ± 1.55 mU βgal/mg of protein, respectively), suggesting that cultures were impermeable to the diffusion of the virus. In contrast, significantly higher levels of β-galactosidase activity were measured in cultures pretreated with C10 (1550 ± 301 mU βgal/mg of protein) and C12-treated cultures (1899 ± 357 mU βgal/mg of protein) (Fig. 1C). These data implied a different mechanism of action for C10 and C12 in comparison with C8.

**C10-and C12-Induced Alterations in Permeability.** The histological appearance of primary WD HAE is shown in Fig. 2A. These cells develop as a well differentiated pseudostratified epithelium consisting of ciliated columnar cells that face the lumen and basal cells. As cultures age, goblet cells may also become apparent, and some multilayering may be seen in the basal cell layer. Note that C10 treatment caused no significant change in the histological appearance of the epithelium, whereas C12 treatment induced intermittent breaks or disruption in the luminal surface (Fig. 2A, see arrows). Measurements of lactate dehydrogenase levels after C10 and C12 exposure revealed no significant increase in lactate dehydrogenase release after C10 treatment, but an increase after C12, indicative of cellular injury (L. G. Johnson, M. K. Vanhook, C. B. Coyne, N. Haykal-Coates, and S. H. Gavett, manuscript submitted for publication).

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**Fig. 1.** Effect of MCFAs on **R**<sub>T</sub> and gene transfer efficiency. A, acute (<5 min) effects of C8, C10, C12 on **R**<sub>T</sub> post-treatment. B, recovery of **R**<sub>T</sub> following removal of C10 and C12. C, AdlacZ transduction following treatment of cultures with vehicle, C8, C10, or C12 48 h postinfection with AdlacZ at an MOI of 100. D, effect of adenovirus serotype-5 fiber knob on AdlacZtransduction following vehicle or MCFA treatment. *, significantly different than vehicle-treated controls (C) and significantly different than cultures treated without AdlacZ fiber knob (D) (p < 0.05), n = 4.
To determine the acute effects of C10 and C12 exposure on permeability, we visualized the penetration of two fluorescently labeled dextrans of 10 and 2000 kDa into live primary WD HAE cells by performing \(xz\)-axis scans by confocal microscopy. Within 15 s of exposure to either C10 or C12, there was cellular uptake of both the 10- and 2000-kDa dextrans into the epithelium (Figs. 2 and 3). C12 treatment allowed permeation of the 10-kDa dextran throughout the epithelium, possibly due to cellular toxicity, whereas 10-kDa dextran in cultures treated with C10 seemed restricted to cells in the luminal portion of epithelium (columnar cells) (Fig. 2A). C12 also increased the permeation of the 2000-kDa dextran to multiple cell types, including columnar and basal cells (Fig. 3A). C10 treatment seemed to restrict permeation of 2000-kDa dextran to columnar cells, whereas basal cells did not seem to be affected (Fig. 3A). Flow of tracer around cells was also detected in C10- and C12-treated cultures, particularly to the 2000-kDa dextran (Fig. 3A, see arrows).

To further assess whether the uptake of dextran was due to cellular or paracellular uptake, we performed \(xy\)-axis scans by confocal microscopy at 60 s after application of either the 10- or 2000-kDa dextran. At 60 s, there was a significant amount of both 10- and 2000-kDa dextran located intracellularly (Fig. 4). These data indicate that both C10 and C12 have widespread effects on the plasma membrane itself, which increases the uptake of dextran into the cytosol. When dextran was applied from the basolateral compartment, with luminal application of vehicle, C10, or C12, minimal cellular fluorescence was detected in C10- and C12-treated cultures at 300 s, but not vehicle (data not shown). These data would indicate that cellular uptake of dextran occurred after C10 and C12 treatment and minimal localization of dextrans into the paracellular region was detected in some cultures (Fig. 4).

To investigate the fate of fluorescently labeled dextran particles during recovery of the epithelium, cultures were washed after the 300-s time point and the fate of the tracer was followed by confocal microscopy for an additional 24 h. By 12 h after treatment, labeled 10- and 2000-kDa dextran was no longer present in cultures treated with C10. The smaller 10-kDa dextran was cleared more slowly from C12-treated cultures resolving within 24 h, whereas the 2000-kDa dextran was cleared within 12 h. These data are not surprising given that the amount of the 10-kDa dextran internalized is much greater than the 2000-kDa dextran (Figs. 2 and 3) and that the permeability to the 10-kDa dextran is higher (Coyne et al., 2000). This suggests that the epithelium after exposure to MCFAs remained intact.

Efficient uptake of Ad particles into host cells requires interaction of the Ad fiber knob with the Coxsackie virus B and Ad2/5 receptor (CAR), which leads to viral internalization and subsequent infection. This receptor is typically localized along the basolateral membrane (Pickles et al., 1998). Because MCFAs seem to have effects on cellular permeability as well as on paracellular, we determined whether the increase in gene transfer efficiency induced by C10 and C12 remained dependent upon interaction of the Ad vector with CAR. Interaction with CAR would not occur if changes in apical membrane permeability mediated enhanced gene transfer.
We incubated cultures treated with vehicle, C10 or C12 with purified recombinant Ad serotype-5 fiber knob, which cross-competes with Ad vector for interaction with the CAR receptor. We found that infection with Ad in vehicle and MCFA-treated cultures could be blocked by excess fiber knob, suggesting that Ad interaction with CAR is required after MCFA treatment (Fig. 1D). Previous studies have demonstrated that polarity is not lost after C10 treatment as evidenced by occludin and ZO-1 localization, and these data indicate that enhancement of gene transfer occurred primarily via the paracellular pathway (Coyne et al., 2000).

**Effect of C10 and C12 on Intracellular Calcium Levels.**

A rapid and sustained increase in Ca\(^{2+}\) measured by Fura-2 fluorescence has been reported as the potential mechanism of action for C10-induced changes in R\(_T\) in Caco-2 cells (Tomita et al., 1995). To determine whether C10 mobilizes calcium from Ca\(^{2+}\) stores in the endoplasmic reticulum (ER), we added apically 5 \(\mu\)M thapsigargin (an ER Ca\(^{2+}\)-ATPase inhibitor) to deplete stores. Subsequent C10 (10 mM) application led to a rapid increase in Ca\(^{2+}\) levels by microfluorometer anlaysis (data not shown), suggesting that C10 may increase Ca\(^{2+}\) via thapsigargin-insensitive Ca\(^{2+}\) stores.

However, the pattern of the sustained increase in Ca\(^{2+}\) induced by C10, which did not return to baseline levels, raised concerns about possible adverse effects of the MCFA on the Fura-2 compound.

To determine whether C10 affected the fluorescence intensity of the individual 340- and 380-nm wavelengths, we measured the effects of C10 under cell-free conditions on 1 \(\mu\)M Fura-2 free acid on a coverslip. C10 induced a significant change in both the 340- and 380-nm wavelengths of the Fura-2 salt, with a more prominent inhibitory effect on the 380-nm wavelength, indicating that the increase in Ca\(^{2+}\) detected after addition of C10 to primary HAE cells was, at least in part, an artifact resulting from the effect of C10 on the Fura-2 fluorescence (data not shown). However, these data did not rule out the potential role of Ca\(^{2+}\) in the mechanism of action of C10.

As an alternative to microfluorometric analysis with Fura-2 for detection of changes in Ca\(^{2+}\) mobilization, we preloaded cultures with Fluo-4 AM followed by confocal microscopic analysis of Ca\(^{2+}\) changes after addition of vehicle (Ca\(^{2+}\)-free HBR), C10, or C12 (in Ca\(^{2+}\)-free HBR) at 0, 15, 30, 60, and 300 s. To prevent Ca\(^{2+}\) influx due to alterations in

![Fig. 3. Imaging of 2000-kD Texas Red dextran permeability in live primary HAE cells. A, primary HAE cultures were exposed to vehicle (medium), 30 mM C10, or 10 mM C12 in nominally Ca\(^{2+}\)-free HBR containing 2 mg/ml Texas Red-labeled 2000-kD dextran and scans taken along the xz-axis at 0, 15, 30, 60, and 300 s following apical application. Images are representative of at least four cultures in each treatment type. B, fate of 10-kD TR-dextran internalized following vehicle or MCFA treatment at 1, 4, 12, and 24 h postexposure. Images are representative of at least six cultures each.](image)

![Fig. 4. Imaging of 10- and 2000-kD Texas Red dextran permeability in live primary HAE cells. Primary HAE cultures were exposed to vehicle (medium), 30 mM C10, or 10 mM C12 in nominally Ca\(^{2+}\)-free HBR containing 2 mg/ml Texas Red-labeled 10- or 2000-kD dextran and scans taken along the xy- and xz-axes at 60 s following apical application. Middle panel, 10-kD dextran; right panel, 2000-kD dextran. Images were captured from of at least four cultures in each treatment group.](image)
membrane permeability, both the apical and basolateral compartments were bathed in Ca\textsuperscript{2+}-free HEPES-buffered saline. Increases in Ca\textsubscript{i}\textsuperscript{2+} were then quantified by measuring the intensity of images. Whereas addition of vehicle to cells caused no change in Ca\textsubscript{i}\textsuperscript{2+}, addition of 30 mM C10 induced a rapid increase in Ca\textsubscript{i}\textsuperscript{2+} levels, which returned to control levels by 300 s after exposure (Fig. 5, A and B). Interestingly, 10 mM C12 had little effect on Ca\textsubscript{i}\textsuperscript{2+} mobilization, indicating a unique mechanism of action for C10 in comparison with C12. These data demonstrated that C10 promoted a rapid Ca\textsubscript{i}\textsuperscript{2+} mobilization, which is suggestive of C10-mediated Ca\textsubscript{2+} release from internal stores.

As a control, the effect of C10 on the free acid of Fluo-4 was determined under cell-free conditions on a coverslip. When 30 mM C10 was added to 1 \mu M Fluo-4 free acid on a coverslip, there was no change in the fluorescence (data not shown), indicating no effects of C10 on the Fluo-4 and that the change in fluorescence induced after addition of 30 mM C10 was due to mobilization of Ca\textsubscript{i}\textsuperscript{2+}.

To determine whether the C10-mediated mobilization of Ca\textsubscript{i}\textsuperscript{2+} was due to release from thapsigargin-sensitive ER stores, we added 5 \mu M thapsigargin to the cultures followed by 100 \mu M UTP to control for full Ca\textsubscript{2+} depletion and then applied C10 (Fig. 6). Under these conditions, C10 did not mobilize Ca\textsubscript{i}\textsuperscript{2+}, whereas controls treated with HBR before C10 released Ca\textsubscript{i}\textsuperscript{2+}. Thus, when thapsigargin-sensitive Ca\textsubscript{i}\textsuperscript{2+} stores were depleted, the C10-mediated mobilization of Ca\textsubscript{i}\textsuperscript{2+} could be blocked, indicating that C10-induced rises in Ca\textsubscript{i}\textsuperscript{2+} resulted from ER Ca\textsubscript{2+} store depletion (Fig. 6).

**Effect of Buffering Ca\textsubscript{2+} on Permeability.** If the action of C10 depends on the rapid increase in Ca\textsubscript{i}\textsuperscript{2+} levels,
then chelation of Ca$_{2+}^+$ should inhibit its effects on the TJ. To chelate Ca$_{2+}^+$, cells were loaded with BAPTA-AM (100 μM) in HBR containing 300 μM Ca$_{2+}^+$ in the basolateral compartment and nominally Ca$_{2+}^+$-free HBR in the apical compartment. Cells were subsequently exposed to 30 mM C10 in nominally Ca$_{2+}^+$-free buffer containing 100 μM BAPTA-AM in the apical and basolateral compartments. The $R_T$ of BAPTA-AM-loaded cultures did not differ significantly from those treated with vehicle before addition of C10. C10 reduced $R_T$ by 94.9 ± 2.5% in vehicle (no BAPTA-AM)-treated cultures, which was not different from the reduction in BAPTA-loaded cells (94.7 ± 1.8%) (Fig. 7A). These data suggested that buffering Ca$_{2+}^+$ to low levels had no effect on C10-mediated changes in $R_T$.

To further test the hypothesis that C10-mediated increases in Ca$_{2+}^+$ were linked with its subsequent effects on $R_T$, we sequentially applied 600 μM lanthanum (La$_{3+}^+$) to block plasma membrane Ca$_{2+}^+$-ATPases, and 5 μM thapsigargin to block ER Ca$_{2+}^+$-ATPases (Paradiso et al., 1995). Under these conditions, the increase in Ca$_{2+}^+$ is sustained, because the Ca$_{2+}^+$-buffering activities at the plasma membrane and at the ER Ca$_{2+}^+$ stores have been inhibited. In cultures where Ca$_{2+}^+$ levels were increased by cotreatment with thapsigargin and La$_{3+}^+$, $R_T$ was 112 ± 11% of vehicle-treated controls, in comparison with 5.9 ± 1.1% in C10-treated cultures with and without La$_{3+}^+$ and thapsigargin pretreatment (Fig. 7A), indicating that the effects of C10 on Ca$_{2+}^+$ may be dissociated from its effects on permeability.

As a control, we measured the effects of this treatment on mobilization of Ca$_{2+}^+$ by confocal microscopy with Fluo-4. Pretreatment of primary HAE cells with BAPTA-AM inhibited the C10-induced increase in Ca$_{2+}^+$ mobilization because there was no change in Fluo-4 intensity after addition of C10 (Fig. 7B). Moreover, treatment with La$_{3+}^+$ and thapsigargin led to a sustained increase in Ca$_{2+}^+$ (Fig. 7B). These data confirmed that the maneuvers to buffer Ca$_{2+}^+$ to high or low levels were successful.

**Effect of Signaling Inhibitors on MCFA-Induced Reduction in $R_T$.** Previously published data suggested that C10-induced increase in Ca$_{2+}^+$ levels may result from PLC activation, which is a necessary step in its long-term effects on $R_T$ (Lindmark et al., 1998). To determine the role of signaling components in the acute effects of C10 on airway permeability, we pretreated primary HAE cultures with...
pharmacological inhibitors of signaling components known to be effectors in TJ regulation in Caco-2 cells: a nonspecific PLC inhibitor (U73122, 50 μM) and phospholipase A2 inhibitor (48/80, 50 μg/ml), an inhibitor of calmodulin (W7, 50 μM), Ca2+/calmodulin-dependent protein kinase inhibitor (KN62, 20 μM), and a nonspecific PKC inhibitor (H7, 50 μM). There was no effect of any inhibitor tested on the C10-induced decreases in $R_T$ (Fig. 8A). Although activation of these signaling cascades cannot be ruled out as a consequence of C10 treatment, they do not seem to play a functional role in the rapid changes in $R_T$ (1–5 min) induced by C10.

Effect of ATP Depletion on $R_T$. Because treatment of Caco-2 cells with C10 has been shown to decrease cellular ATP levels, we investigated whether ATP depletion could induce the same effect on $R_T$ as C10 (Lindmark et al., 1998).

We depleted ATP levels by inhibiting both the oxidative and glycolytic metabolic pathways in primary HAE cells. Although this depletion did lead to a reduction in $R_T$, the kinetics of these changes was distinct from that of either C10 or C12. C10 and C12 both exert their maximal effects on $R_T$ by 2 to 5 min post-treatment, whereas the maximal reduction in $R_T$ induced by depletion of ATP did not occur until after 90 min of treatment (Fig. 8B). Therefore, the depletion of ATP by C10 and C12 does not seem to play a functional role in the acute effects on TJ barrier function.

Effect of C10 and C12 on Structural Components of the TJ. We evaluated whether MCFAs that modulate airway permeability do so by their effects on key structural components of the TJ. Because JAM has been speculated to play a role in maintaining junctional integrity, we investigated the effect of MCFA exposure of primary HAE cells on JAM distribution. Although JAM was clearly redistributed after exposure to either C10 or C12, ZO-1 distribution remained relatively unchanged (Fig. 9). C10 and C12 also induced a redistribution of actin (Fig. 10A), which has been shown previously to interact directly with the TJ via its interaction with ZO-1 (Fanning et al., 1998, 2002). Image analysis of

![Fig. 9](image)

**Fig. 9.** Localization of ZO-1 and JAM following MCFA exposure. Immunofluorescent staining for ZO-1 and JAM in primary HAE cells exposed to vehicle, 30 mM C10, or 10 mM C12 for 5 min. JAM staining is in green, ZO-1 is in red, and the merged image is at the bottom. Colocalization appears as yellow. Images are representative of at least three cultures each.

![Fig. 10](image)

**Fig. 10.** MCFA-induced redistribution of actin and β-catenin. A, primary HAE cultures were treated with vehicle, 30 mM C10, or 10 mM C12 and stained for actin. Additionally, cells were pretreated with Bapta-AM before C10 exposure or La3⁻ and thapsigargin and stained for actin. B, immunofluorescent localization of β-catenin in primary HAE cells exposed to vehicle, 30 mM C10, or 10 mM C12 for 5 min. Blue staining represents DAPI-stained nuclei, and red staining represents β-catenin staining. Data are representative of imaging of at least three cultures each.
relative fluorescence intensity revealed that both C10 and C12 caused redistribution of actin, with 39 ± 3.3% less intensity for C10 and 38 ± 5% for C12 in comparison with vehicle-treated controls. This redistribution was not blocked by preincubation of cultures with BAPTA-AM and could not be replicated by treatment of naïve cells with La3+ and thapsigargin (Fig. 10A). The data showing no changes in actin distribution after incubation with La3+ and thapsigargin suggested that the effects of C10 and C12 on actin are Ca2+-independent.

The direct interaction of actin with the catenin proteins and regulation of claudin-1 by β-catenin (Miwa et al., 2001) suggests that MCFA exposure of primary HAE cells may also lead to reorganization of members of the AJ. Because β-catenin functions both as a link to the cytoskeleton as well as a transcriptional regulator, we investigated the effects of C10 and C12 on this protein component. Whereas vehicle-treated cultures maintained β-catenin localization circumscribing the cell, HAE cells exposed to either C10 or C12 showed modest disruption of β-catenin distribution (Fig. 10B). Image analysis of relative fluorescence intensity showed 26 ± 3 and 32 ± 5% less staining intensity in C10- and C12-treated cultures, respectively, compared with vehicle-treated controls. Although both C10 and C12 caused reorganization of β-catenin, neither agent had any effect on the localization of E-cadherin (data not shown).

The claudin family of transmembrane proteins clearly plays a significant role in the maintenance of junctional integrity (Tsukita et al., 2001). As shown in Fig. 11, primary HAE cells express both claudin-1 and -4 by immunofluorescent staining and confocal microscopy. Moreover, claudin-1 and -4 were redistributed immediately after C10 and C12 treatment (Fig. 11). Changes in claudin distribution correlated with a decrease in staining intensity of 72 ± 8 and 78 ± 4% for C10 and C12, respectively, compared with vehicle-treated controls. Because claudins seem to function independently of Ca2+, the effect of C10 on this family of proteins suggests a Ca2+-independent link to its effects on the TJ.

Discussion

The ability of the MCFAs C10 and C12 to enhance drug absorption and in more recent studies, absorption of Ad vectors, has emphasized the therapeutic potential of these compounds. A role for Ca2+ regulation of transepithelial permeability has been suggested as a mechanism for these compounds. Alterations in either extracellular or intracellular Ca2+ levels have a pronounced effect on TJ barrier function and may contribute to the relative permeability of the junction. It is therefore not surprising that agents that lead to alterations in Ca2+ may have effects on both RT and solute permeability. However, the data presented here indicate that although C10 and C12 have similar effects on the protein components of the TJ, their effects on Ca2+ levels diverge. This incongruity would suggest that C10 and C12 have dissimilar mechanisms, and/or that the effect of C10 on Ca2+ is a secondary response not associated with its effects on permeability.

The RT of primary HAE cells was significantly reduced within seconds after exposure to either C10 or C12, whereas treatment of cultures with C8 did not have any effect on RT (Fig. 1A). The decrease in RT induced by C10 and C12 correlated with a significant enhancement of AdlacZ transduction, indicating that the barrier function of the TJ was altered after MCFA exposure, whereas C8 had no effect on this parameter (Fig. 1C). The increase in transduction efficiency after pretreatment of cultures with C10 and C12 indicate considerable effects of these agents on the airway permeability to macromolecules, because Ad vectors are approximately 2000 to 4000 kDa. C8 had no effects on either RT or paracellular permeability and thus was unable to enhance diffusion of an Ad vector to the basolateral surface. The significant effects of both C10 and C12 on permeability were reversible and returned to baseline levels within 24 h of treatment (Fig. 1B).

We further explored the effects of C10 and C12 on airway epithelial permeability with live cell imaging of fluorescently labeled dextrans by confocal microscopy (Figs. 2 and 3). Surprisingly, C10 and C12 induced cellular uptake of both low (10 kDa) and high (2000 kDa) molecular weight dextrans, which took greater than 4 h to clear for C10 and greater than 12 h to clear when induced by C12. C10 induced a relatively specific uptake in columnar cells within the epithelium, whereas C12-induced uptake into both columnar and basal cells. The cellular uptake of dextran in C12-treated cultures may be exaggerated by the relative toxicity induced by this agent. Paracellular flow of dextrans was also increased after C10 and C12 treatments (Figs. 2A and 3A, see arrows). Although the images would suggest that the majority of dextran uptake seemed to be cellular, the retention of dextrans within cells prevents an accurate assessment of cellu-
ular uptake relative to the amount of paracellular flow (Fig. 4). Therefore, measurements of mannitol or dextran flux immediately after MCFA-treatment would not detect uptake into cellular compartments, rather these measurements of solute permeability would exclusively reflect paracellular flow.

Because of the increased cellular uptake of dextran across the apical membrane, we tested whether CAR-dependent entry of Ad was required for transduction of WD primary HAE cells after treatment with C10 or C12. If increased apical membrane permeability increased Ad uptake, competition with Ad fiber knob for CAR binding should not inhibit Ad transduction. As shown in Fig. 1D, incubation of C10- and C12-treated cells with Ad vector in the presence of excess fiber knob protein inhibited Ad-mediated transduction. Because CAR has been localized to the basolateral membrane of WD HAE cells (Pickles et al., 1998), these data suggest that the enhancement of Ad gene transfer occurred predominantly by enhancing paracellular permeability. Increased cellular permeability played a much smaller role in enhancing gene transfer.

Our data indicated that measurements of the effects of C10 on Ca$^{2+}$ by Fura-2 were complicated by interference of C10 and C12 (data not shown) with Fura-2 fluorescence, particularly its effects on the 380-nm wavelength even at doses as low as 0.3 mM (data not shown). However, our studies with Fluo-4-loaded cells support previous reports that C10 application leads to a rapid and sustained increase in Ca$^{2+}$ (Tomita et al., 1995, 1996; Lindmark et al., 1998), resulting from the release of thapsigargin-sensitive ER Ca$^{2+}$ stores (Figs. 5 and 6). Although C10 does lead to changes in Ca$^{2+}$, correlating temporally with changes in $R_T$, our data demonstrated that this effect may be dissociated from C10 effects on permeability in primary HAE cells. When Ca$^{2+}$ is chelated with BAPTA-AM, C10 and C12 maintain their ability to rapidly decrease $R_T$ (Fig. 7A), consistent with a Ca$^{2+}$-independent mechanism. Additionally, mimicking the rapid, sustained increase in Ca$^{2+}$ induced by C10 by treatment of cells with thapsigargin and La$^{3+}$ had no effect on $R_T$ (Fig. 7A). These studies combined confirm that the effects of C10 and C12 on permeability are independent of changes in Ca$^{2+}$.

Although ATP levels have been shown to be decreased after application of C10, this is likely a secondary response, which may contribute to the long-term, but not acute, effects of C10 on $R_T$ and permeability. This is supported by the differences in the effects of ATP depletion and C10 on $R_T$, where greater than 15 min was required for ATP depletion to decrease $R_T$ compared with C10-induced effects on $R_T$ occurring within seconds and reaching maximum effects in minutes (Fig. 8). These data support previously published data showing reversible long-term decreases in $R_T$ after ATP depletion (Canfield et al., 1991).

The similar effects of C10 and C12 on protein components of the TJ provide further evidence that C10 acts via a Ca$^{2+}$-independent pathway and likely acts upon the same protein components as C12 in airway epithelia. Although C12 did not lead to increases in Ca$^{2+}$, both C10 and C12 caused redistribution of the same TJ protein components, the claudins and JAM. The claudin family of transmembrane proteins seems to exert effects on TJ integrity independently of Ca$^{2+}$ (Kubota et al., 1999). The reorganization of claudins-1 and -4 may account for the almost immediate effects of both C10 and C12 on $R_T$ and permeability (Fig. 11). This would suggest that these proteins are unaffected by agents that alter junctional integrity via a Ca$^{2+}$-dependent cascade.

Although C10 and C12 do not significantly affect the distribution of ZO-1, both led to significant relocalization of JAM, which has been shown to interact with ZO-1 (Fig. 9) (Bazzoni et al., 2000; Ebnet et al., 2000). Because JAM has been implicated in the resealing of the junction (Liu et al., 2000), this suggests that the redistribution of JAM may contribute to the MCFA-induced effects on paracellular permeability.

Actin filaments play a central role in cellular architecture and have been shown to bind directly to TJ-associated proteins. Both C10 and C12 caused redistribution of actin in primary HAE cultures. Previously published data in Caco-2 cells suggested that the initial increase in Ca$^{2+}$ levels induced by C10 causes the reorganization of actin filaments (Sakai et al., 1998). However, when primary HAE cultures were treated with lanthanum and thapsigargin to mimic the rapid and sustained increase in Ca$^{2+}$ levels induced by C10, there was no effect on actin distribution (Fig. 10A). Furthermore, although C12 had minimal effects on Ca$^{2+}$ levels, it induced a similar level of reorganization as C10, indicating Ca$^{2+}$-independent reorganization. C10 and C12 also affected a component of the adherens junction, β-catenin, which has also been shown to regulate claudin-1 gene expression (Miwa et al., 2001).

The results of this study suggest that both C10 and C12 exert their acute effects on permeability via a Ca$^{2+}$-independent mechanism. Although exposure of primary HAE cells to C10 caused a rapid increase in Ca$^{2+}$ levels, exposure to C12 had little to no effect on Ca$^{2+}$ levels. In addition, inhibitors of Ca$^{2+}$-dependent signaling cascades had no effect on the C10-induced effects on $R_T$, nor did blocking the rise in Ca$^{2+}$ with BAPTA-AM, or mimicking the increase with thapsigargin and lanthanum. However, both C10 and C12 caused reorganization of JAM, actin, claudins-1 and -4, suggesting that both agents exert their effects on the TJ via direct or indirect effects on these protein components. Thus, C10 and C12 may act via similar Ca$^{2+}$-independent mechanisms in human primary airway cells to enhance permeability.

The effects of C10 and C12 on apical membrane permeability and cellular uptake are more of an enigma. Although disruption of apical membrane lipids with loss and collapse of the apical domain might occur, preservation of ZO-1 staining (Fig. 9) and previous data demonstrating that C10 does not disrupt occludin distribution in WD HAE cultures (Coyne et al., 2000) suggest that the polarity of these cells remains intact after C10 and C12 treatment. Clearly, an immediate increase in apical membrane permeability occurs that is restricted to the apical pole by a functional TJ. The cellular retention of dextran for extended periods (4–12 h) in lumenchacing columnar cells is consistent with this notion. When this observation is coupled with the inhibition of Ad-mediated transduction by excess fiber knob and the immediate changes in claudins-1, -4, and JAM localization, these data suggest that an increase in paracellular permeability is the primary method of C10- and C12-induced enhancement of Ad-mediated gene transfer.

Understanding how MCFAs enhance permeability to permit more efficient transduction of airway cells is important because it may allow for more accurate predictions of toxicities induced by this approach in human subjects.
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