Riboflavin uptake by human-derived colonic epithelial NCM460 cells

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Riboflavin uptake by human-derived colonic epithelial NCM460 cells. Am. J. Physiol. Cell Physiol. 278: C270–C276, 2000.—Normal microflora of the large intestine synthesize a number of water-soluble vitamins including riboflavin (RF). Recent studies have shown that colonic epithelial cells posses an efficient carrier-mediated mechanism for absorbing some of these micronutrients. The aim of the present study was to determine whether colonic cells also posses a carrier-mediated mechanism for RF uptake and, if so, to characterize this mechanism and study its cellular regulation. Confluent monolayers of the human-derived nontransformed colonic epithelial cells NCM460 and [3H]RF were used in the study. Uptake of RF was found to be 1) appreciable and temperature and energy dependent; 2) Na+ independent; 3) saturable as a function of concentration with an apparent Km of 0.14 µM and Vmax of 3.29 pmol·mg protein⁻¹·min⁻¹; 4) inhibited by the structural analogs lumiflavin and lumichrome (Ki of 1.8 and 14.1 µM, respectively) but not by the unrelated biotin; 5) inhibited in a competitive manner by the membrane transport inhibitor amiloride (Ki = 0.86 mM) but not by furosemide, DIDS, or probenecid; 6) adaptively regulated by extracellular RF levels with a significant and specific upregulation and downregulation in RF uptake in RF-deficient and oversupplemented conditions, respectively; and 7) modulated by an intracellular Ca²⁺/calmodulin-mediated pathway. These studies demonstrate for the first time the existence of a specialized carrier-mediated mechanism for RF uptake in an in vitro cellular model system of human colonocytes. This mechanism appears to be regulated by extracellular substrate level and by an intracellular Ca²⁺/calmodulin-mediated pathway. It is suggested that the identified transport system may be involved in the absorption of bacterially synthesized RF in the large intestine and that this source of RF may contribute toward RF homeostasis, especially that of colonocytes.

The vitamin is involved in key metabolic reactions including carbohydrate, amino acid, and lipid metabolism and in the conversion of folic acid and pyridoxine into their coenzyme forms (2). RF deficiency occurs in humans and leads to a variety of clinical abnormalities, including degenerative changes in the nervous system, endocrine dysfunction, anemia, and skin disorders (6, 9, 12, 13, 18).

Humans and other mammals have lost their ability to synthesize RF and thus must obtain the vitamin from exogenous sources via intestinal absorption. The intestine is exposed to RF from two sources: 1) the diet, and 2) the bacterially synthesized RF in the large intestine. Absorption of dietary RF has been extensively studied over the past three decades using a variety of intestinal preparations, including gut everted sacs, perfused intestinal segments, purified intestinal membrane vesicles, and cultured intestinal epithelial cells (1, 3, 15, 19–24). Results of these studies have shown that RF uptake takes place mainly in the proximal part of the small intestine and involves a specialized, carrier-mediated mechanism.

With regard to the bacterially synthesized RF, it has been known that the normal microflora of the large intestine synthesize considerable amounts of RF and that a significant portion of this RF exists in the free form, i.e., available for absorption (7, 17). The amount of RF provided through this source varies depending on the diet, being significantly higher following consumption of a vegetable-based diet compared with a meat-based diet (7). Studies in humans and rats have shown that the large intestine is capable of absorbing a large amount of luminally introduced RF (8, 28). Nothing, however, is known about the mechanism and regulation of the RF uptake process in the large intestine. The existence of a specialized, carrier-mediated mechanism for RF uptake in the large intestine is possible in light of the recent findings of specialized carrier-mediated systems for uptake of other water-soluble vitamins that are synthesized by the normal microflora of the large intestine, such as folate, biotin, and pantothenic acid (10, 26). Our aim in this study was, therefore, to test this possibility using the human-derived colonic epithelial cell line NCM 460 (16) as an in vitro experimental model system. These cells were chosen because they possess characteristics similar to that of the native colonocytes, including similar uptake mechanisms (4,
Our study demonstrates the existence of a specialized, carrier-mediated mechanism for RF uptake in NCM460 cells and provides evidence to suggest that the process is regulated by extracellular substrate levels and by an intracellular Ca$^{2+}$/calmodulin-mediated pathway.

MATERIALS AND METHODS

$[^3H$-(G)]$RF (sp act 27.5 Ci/mmol; radiochemical purity determined by the manufacturer and by us was >98%) was purchased from Moravek Biochemicals (Brea, CA). Trypsin, fetal bovine serum (FBS), and other cell culture materials were obtained from Irvine Scientific (Santa Ana, CA). Ham’s F-12 growth medium was obtained from Sigma Chemical. Unlabeled RF and all other chemicals and reagents were purchased from Moravek Biochemicals (Brea, CA). Trypsin, fetal bovine serum (FBS), and other cell culture materials were obtained from commercial sources and were of analytical quality.

The human-derived nontransformed colonic epithelial cell line NCM460 was routinely maintained in M3:10 medium (INCELL, San Antonio, TX) but was shifted to Ham’s F-12 culture medium supplemented with 20% (vol/vol) FBS and antibiotics for the experiments described in this study. NCM460 cells were used between passages 29 and 39 in this study. The cells were grown in 75-cm$^2$ plastic flasks (Costar) at 37°C in a 5% CO$_2$-95% air atmosphere with media changes every 4 days. NCM460 cells were subcultured by trypsinization with 0.05% trypsin and 0.9 mM EDTA in Ca$^{2+}$- and Mg$^{2+}$-free PBS and plated onto 12-well plates at a concentration of 5 × 10$^5$ cells/well. Uptake of RF was studied 2-4 days following confluence. Cell growth was observed by periodic monitoring with an inverted microscope. Cell viability was tested by the trypan blue dye exclusion method and found to be >95%.

Uptake experiments were performed at 37°C, unless otherwise stated. Incubation was performed in Krebs-Ringer buffer containing (in mM) 123 NaCl, 4.93 KCl, 1.23 MgSO$_4$, 0.85 CaCl$_2$, 5 glucose, 5 glutamine, 10 HEPES, and 10 MES (pH 7.0), unless otherwise stated. $[^3H]RF$ was added to the incubation buffer at the onset of experiments and uptake was terminated after 3 min of incubation (unless otherwise specified) by the addition of 1 ml of ice-cold buffer followed by immediate aspiration. The monolayers were rinsed twice with ice-cold buffer and digested with 1 ml of 1 N NaOH, neutralized by HCl, and then counted for radioactivity in a liquid scintillation counter. Protein contents of cell digests were estimated on parallel wells by a Bio-Rad protein assay kit. Uptake data are means ± SE of multiple separate monolayers performed on at least two different occasions and are expressed in femtomoles or picomoles per milligram protein per unit time. P values of experimental vs. simultaneously performed control groups were calculated using the Student’s t-test. Kinetic parameters of RF uptake, i.e., maximal velocity ($V_{max}$) and the apparent Michaelis constant ($K_m$), were calculated using a computerized nonlinear regression analysis program of the Michaelis-Menten equation as described previously (29).

In the study examining the effect of growing cells in the presence of different RF levels in the growth medium on subsequent uptake of $[^3H]RF$, cells were incubated for 24 h in a control growth medium (a medium that contains a total of 38.15 ng/ml RF, of which 38 ng/ml RF was added to the DMEM cultured medium and the rest (~0.15 ng/ml) was contributed by the added 5% FBS), a RF-deficient medium (no RF added to the growth medium except that contributed by 5% FBS), and a RF oversupplemented medium (which contains 50-fold the normal amount of RF added to the control growth medium, i.e., 0.15 μg/ml). When examining the effect of growing the cells in a medium containing different levels of the RF structural analog lumiflavin (LF), the 38 ng/ml of RF added to the normal control growth medium was replaced with an equal amount of LF (“control LF state”). The “LF-deficient state” contained no added LF, whereas the “LF-oversupplemented state” contained 10-fold the normal amount of LF added to the control LF state growth medium (i.e., 1.9 μg/ml). Therefore, in all the studies with LF levels, the RF level was ~0.15 ng/ml, which is provided by the added 5% FBS. No change in cell viability or appearance was observed compared with normal control NCM460 monolayers in all the studies with the different RF and LF levels.

On examining the metabolic form of the $^3$H radioactivity taken up by NCM460 cells following incubation with $[^3H]RF$, silica-gel-precoated TLC plates and a solvent system of ethanol and water (9:1 vol/vol) were used as described previously (5).

RESULTS

Uptake of RF with time, and effect of temperature, incubation buffer pH, and Na$^+$. Uptake of low (5.5 nM) and high (1 μM) concentrations of RF by NCM460 cells was examined as a function of time. At both concentrations, RF uptake was linear over the entire 5 min of incubation and occurred at a rate of 0.032 and 0.870 pmol·mg protein$^{-1}$·min$^{-1}$, respectively (Fig. 1). A 3-min
incubation time was used as the standard incubation time in all subsequent studies.

The metabolic form of the $^3$H radioactivity taken up by NCM460 cells following a 3-min incubation with 17 nM $[^3H]$RF was examined using a TLC system described in MATERIALS AND METHODS. The result showed that the majority (97%) of the transported $^3$H radioactivity was in the form of intact RF. We also examined the effect of incubation temperature on the uptake of RF (5.5 nM) and found significant ($P < 0.01$) decreases in RF uptake on decreasing buffer temperature from 37 to 22 to 4°C (129 ± 3.14, 78.16 ± 3.05, and 36.48 ± 4.24 fmol·mg protein$^{-1}$·3 min$^{-1}$, respectively).

The effect of varying incubation buffer H$^+$ concentration ([H$^+$]$^-$) and [Na$^+$] on the uptake of RF was also tested. [H$^+$] was varied by changing buffer pH over the range 5.0–8.0. The results showed that lowering the incubation buffer pH from 8.0 to 7.0 leads to an increase in RF (5.5 nM) uptake; no further increase, however, was observed at lower pH values (65.79 ± 6.64, 105.62 ± 5.26, 133.50 ± 4, 131.55 ± 2.59, 133 ± 4.68, and 141.98 ± 3.31 fmol·mg protein$^{-1}$·3 min$^{-1}$ at buffer pH 8.0, 7.5, 7.0, 6.5, 6.0, and 5.0, respectively). With regard to an effect of Na$^+$, this was tested by replacing the [Na$^+$] in the incubation medium isosmotically with Li$^+$ or NH$_4^+$ on RF (5.5 nM) uptake. The results showed no inhibition in RF uptake on such replacement [121 ± 3.2, 127.19 ± 8.98, and 135.17 ± 4.11 fmol·mg protein$^{-1}$·3 min$^{-1}$ in the presence of Na$^+$ (control), Li$^+$, and NH$_4^+$, respectively]. This is in contrast to uptake of the unrelated biotin by these cells, which showed its known Na$^+$ dependence [14.77 ± 0.84, 6.60 ± 0.16, and 5.36 ± 0.43 fmol·mg protein$^{-1}$·3 min$^{-1}$ in the presence of Na$^+$ (control), Li$^+$, and NH$_4^+$, respectively]. We also examined the effect of pretreating (for 30 min) the cells with the Na$^+$/K$^+$-ATPase inhibitor ouabain (1 and 10 mM) on the uptake of RF (5.5 nM). The results showed no effect of such treatment on the vitamin uptake (128.3 ± 3.4, 134.7 ± 4.9, and 124.5 ± 8.6 fmol·mg protein$^{-1}$·3 min$^{-1}$ in the absence and presence of 1 and 10 mM ouabain, respectively).

Uptake of RF as a function of concentration. The initial rate of RF uptake (3 min) by NCM460 cells was examined as a function of increasing the substrate concentration in the incubation medium (5.5–1,000 nM). Uptake was found to include a saturable component. Uptake by this component was determined by subtracting diffusion uptake (calculated from the slope of the line between uptake at high pharmacological concentration of 100 µM and the point of origin) from total uptake. Kinetic parameters of the saturable component, i.e., the apparent $K_m$ and $V_{max}$, were then calculated as described in MATERIALS AND METHODS and found to be 0.14 ± 0.004 µM and 3.29 ± 0.58 pmol·mg protein$^{-1}$·3 min$^{-1}$, respectively (Fig. 2).

Specificity of the RF uptake system: effect of RF structural analogs on $[^3H]$RF uptake. The effect of different concentrations of the structural analogs LF and lumichrome (LC) and that of the unrelated biotin (1 mM) on the initial rate of $[^3H]$RF (5.5 nM) uptake was examined. The results showed that both LF and LC cause a concentration-dependent inhibition in $[^3H]$RF uptake with an inhibition constant ($K_i$) of 1.8 and 14.1 µM, respectively (Fig. 3). The unrelated biotin, on the other hand, showed no effect on RF uptake (144.2 ± 2.8 and 142 ± 2.2 fmol·mg protein$^{-1}$·3 min$^{-1}$ for control and in the presence of biotin, respectively).

Energy requirement of the RF uptake process and effect of membrane transport inhibitors. In this study, we examined the effect of pretreating cells for 30 min with the metabolic inhibitors p-cholomercuprophyleylsulfonate (p-CMPS; 1 mM), dinitrophenol (DNP, 10 mM), and azide (10 mM) on the uptake of RF (5.5 nM). The results showed significant ($P < 0.01$ for all) inhibition in RF uptake by all tested compounds (128.3 ± 3.4, 47.8 ± 2.74 ± 1.53, and 91.8 ± 8.5 fmol·mg protein$^{-1}$·3 min$^{-1}$ for control and on pretreatment with p-CMPS, DNP, and azide, respectively).

In another study, we examined the effect of the membrane transport inhibitors furosemide, DIDS, probenecid, and amiloride (all at 1 mM) on the uptake of RF (5.5 nM). The results showed that, with the exception of amiloride, which caused a significant ($P < 0.01$) inhibition in RF uptake, none of the other compounds inhibited RF uptake (135.7 ± 2.4, 137.6 ± 2.3, 139.2 ± 10.4, 145.28 ± 3.9, and 73.1 ± 5.4 fmol·mg protein$^{-1}$·3 min$^{-1}$ for control and in the presence of furosemide, DIDS, probenecid, and amiloride, respectively). The inhibitory effect of amiloride (1 mM) was observed both in the presence and absence of Na$^+$ in the incubation medium (134.55 ± 10.9, 45.76 ± 3.59, and 33.59 ± 2.22 fmol·mg protein$^{-1}$·3 min$^{-1}$ for control, in the presence of Na$^+$ and amiloride, and in the absence of Na$^+$ and presence of amiloride, respectively). The effect of amiloride (1 mM) was investigated further by the Dixon method to determine the type of the inhibition and inhibition constant. The result showed the inhibition to be competitive in nature with a $K_i$ of 0.86 mM (Fig. 4).
examined the effect of growing NCM460 cells for 24 h under RF-deficient and RF-oversupplemented conditions on the subsequent uptake of the vitamin. The results were compared with that of control (i.e., cells grown in RF-sufficient medium). Growing the cells in RF-deficient medium was found to lead to significant (P < 0.01) upregulation of [3H]RF (5.5 nM) uptake compared with control cells, whereas growing them under RF oversupplemented conditions leads to a significant downregulation in uptake (182.8 ± 3.7, 124.7 ± 2.2, and 72.9 ± 1.1 fmol·mg protein^{-1}·3 min^{-1}, respectively). Uptake of the unrelated biotin (6.4 nM), however, was not affected by growing the cells under such conditions (34.4 ± 0.8, 35.8 ± 0.1, and 34.6 ± 1.2 fmol·mg protein^{-1}·3 min^{-1} for cells grown under control, RF-deficient, and RF oversupplemented conditions, respectively). To better define the mechanism involved in this adaptive regulation of RF uptake, we determined whether the effect of substrate level is mediated via changes in the V_{max} and/or the apparent K_{m} of the RF uptake process. This was performed by examining the initial rate of [3H]RF uptake as a function of concentration in cells grown under control, RF-deficient, and RF oversupplemented conditions. Kinetic parameters were then determined as described in MATERIALS AND METHODS. The results (Fig. 5) showed a significant (P < 0.01) increase in the V_{max} of the RF uptake process in cells grown under RF-deficient conditions compared with control, while a significant (P < 0.01) decrease in V_{max} was observed in cells grown under RF oversupplemented conditions (V_{max} of 182.8 ± 3.7, 124.7 ± 2.2, and 72.9 ± 1.1 fmol·mg protein^{-1}·3 min^{-1} for control and in cells grown under RF-deficient and oversupplemented conditions, respectively). On the other hand, no significant change in the apparent K_{m} of the RF uptake system was observed under the...
different conditions (apparent K_m of 0.14 ± 0.01, 0.15 ± 0.02, and 0.18 ± 0.03 µM for control and in cells grown under RF-deficient and oversupplemented conditions, respectively). In another study, we examined the effect of adding (at the time of media change, i.e., 24 h before uptake studies) the transcription inhibitor actinomycin D (50 µM) on [3H]RF uptake by cells grown in RF-deficient and RF-oversupplemented growth media. Actinomycin D did not affect RF uptake by cells grown in RF-oversupplemented medium (48.51 ± 1.3 and 48.34 ± 1.6 fmol·mg protein⁻¹·3 min⁻¹ in the absence and presence of actinomycin, respectively); however, actinomycin D caused a significant (P < 0.01) inhibition in RF uptake by cells grown in RF-deficient medium (147.28 ± 4.1 and 117.33 ± 4.0 fmol·mg protein⁻¹·3 min⁻¹ in the absence and presence of actinomycin D, respectively).

In a related study, we examined the effect on [3H]RF uptake of replacing the added RF in the study described above with identical amounts of its structural analog LF as described in MATERIALS AND METHODS. The results showed that growing the cells for 24 h under an LF-deficient state leads to a significant (P < 0.01) increase in [3H]RF uptake compared with cells grown under an LF control state, whereas growing them under an LF-oversupplemented state leads to a significant (P < 0.01) decrease in [3H]RF uptake (131.8 ± 2.73, 112 ± 3.58, and 76.2 ± 3.58 fmol·mg protein⁻¹·3 min⁻¹ for LF-deficient, LF control, and LF-oversupplemented states, respectively).

Regulation of RF uptake by colonic NCM460 cells: possible role of intracellular regulatory pathways. In this study, we examined the possible involvement of Ca²⁺/calmodulin- and protein kinase C (PKC)-mediated pathways in cellular regulation of the RF uptake process of NCM460 cells. The study was performed using specific modulators of these pathways. The role of the Ca²⁺/calmodulin-mediated pathway in the regulation of RF uptake by NCM460 cells was investigated by examining the effect of pretreating (for 1 h) the cells with the Ca²⁺/calmodulin inhibitor calmidazolium on the uptake of RF (5.5 nM). The results showed significant (P < 0.01) and concentration-dependent inhibition in RF uptake by this compound (108 ± 2.5, 71.5 ± 12.6, and 46.2 ± 8.2 fmol·mg protein⁻¹·3 min⁻¹ for control (containing DMSO) and in the presence of 10 and 50 µM of calmidazolium, respectively). Similarly, the Ca²⁺/calmodulin inhibitor trifluoperazine (TFP) also caused a significant (P < 0.01) and concentration-dependent inhibition in RF uptake (128.7 ± 2.4, 99.4 ± 5.7, and 64.9 ± 4.9 fmol·mg protein⁻¹·3 min⁻¹ for control and in cells pretreated with 50 and 100 µM TFP, respectively).

In another experiment, we determined whether the inhibitory effect of calmidazolium on RF uptake is mediated via an effect on the apparent K_m and/or the V_max of the uptake process. This was done by examining the effect of 10 µM calmidazolium on the uptake of RF as a function of concentration. The results (Fig. 6) showed a significant (P < 0.01) decrease in the V_max of RF uptake and a significant (P < 0.01) increase in apparent K_m (V_max of 2.61 ± 0.042 and 1.93 ± 0.092 pmol·mg protein⁻¹·3 min⁻¹; apparent K_m of 0.135 ± 0.007 and 0.22 ± 0.03 µM for control and calmidazolium-pretreated cells, respectively).

The possible role of a PKC-mediated pathway in the regulation of RF uptake by NCM460 cells was also examined by investigating the effect of pretreating the cells with modulators of this pathway on RF uptake. The results showed that neither activation (with the use of phorbol 12-myristate 13-acetate (PMA; 10 µM) and sn-1,2-dioctanoylglycerol (DAG; 10 µM)) nor inhibition (with the use of chelerythrine) of this pathway causes significant changes in RF uptake (141.6 ± 2.6, 149.3 ± 3.1, 152 ± 6, and 150.7 ± 7.4 fmol·mg protein⁻¹·3 min⁻¹ for control and in the presence of PMA, DAG, and chelerythrine, respectively).

DISCUSSION

The aim of the present study was to test for the existence of a transport mechanism for the water-soluble vitamin RF in an in vitro model system of human colonocytes, the NCM460 cells, and to determine the characteristics and regulation of any existing system. The results showed that uptake of RF by these cells is temperature dependent, with minimal metabolic alteration in the transported substrate. Uptake was Na⁺ independent, as indicated by lack of inhibition in the vitamin uptake on replacing Na⁺ with other monovalent cations and by the lack of effect on RF uptake of pretreatment of the cells with the Na⁺-K⁺-ATPase inhibitor ouabain. Incubation buffer pH had a limited effect on RF uptake over the pH range of 5 and 7, but uptake decreased at higher pHs. The cause of this decrease is not clear and requires further investigation.

The uptake process of RF by NCM460 cells was found to involve a specialized, high-affinity carrier-mediated system. This conclusion was supported by observations...
of saturation in RF uptake as a function of concentration (apparent $K_m = 0.14 \, \mu M$) and by the inhibition in RF uptake by the structural analogs LF and LC ($K_i$ values of 1.8 and 14.1 $\mu M$, respectively). The closer agreement of the $K_i$ value of LF to the apparent $K_m$ of the RF uptake process compared with the $K_i$ value of LC suggests that replacing the ribityl side chain at nitrogen-10 of the isoalloxazin ring by a smaller group (i.e., -CH$_3$) does not markedly affect the ability of the new compound (i.e., LF) to interact with the vitamin membrane transporter. On the other hand, complete removal of the side chain from position 10 of the isoalloxazin ring may lead to a decrease in the ability of the new compound (i.e., LC) to interact with the RF membrane transporter.

The RF uptake process of NCM460 cells was energy dependent, as indicated by the significant inhibition in the substrate uptake by metabolic inhibitors. The study on the effect of membrane transport inhibitors on RF uptake produced interesting findings. Although furosemide, DIDS, and probenecid had no effect on RF uptake, competitive inhibition of RF uptake was observed by amiloride. This inhibition was observed whether the experiment was performed in the presence or absence of Na$^+$ in the incubation medium, suggesting that the effect of amiloride is not mediated through an inhibition of its main target, i.e., the Na$^+$/H$^+$ exchanger.

The above-described findings on the existence of a specialized, carrier-mediated mechanism for RF uptake in cultured human colonocytes are similar to those seen previously with human and animal small intestinal preparations (1, 3, 15, 19–24). This suggests that the same uptake mechanism may be operating in the large intestine to absorb the bacterially synthesized RF. After the identification of a carrier-mediated mechanism for RF uptake in cultured human colonocytes, we investigated possible regulation of the identified uptake process by extracellular RF levels. The nutritional importance of addressing this issue stems from the finding that intraluminal RF levels in the large intestine vary depending on the type of the diet consumed, being higher following a vegetable-based diet compared with a meat-based diet (7). The results showed that uptake of [3H]RF by cells grown in a RF-deficient medium is significantly higher than uptake by cells grown in control (i.e., RF-sufficient) medium. On the other hand, uptake by cells grown in a RF-oversupplemented medium was significantly lower than that of control. This effect of extracellular RF substrate levels on RF uptake appears to be specific in nature, since uptake of the unrelated biotin was found to be similar under the three differing RF growth conditions. The adaptive regulatory effect of extracellular RF level on RF uptake was mediated through a significant increase in the $V_{max}$ of the RF uptake process, with no significant changes in the apparent $K_m$. These findings suggest that these adaptive regulatory effects are mediated via changes in the number (and/or activity) and not the affinity of the RF uptake system. The observation that the transcription inhibitor actinomycin D caused significant inhibition in the induced RF uptake in cells grown under RF-deficient conditions suggests possible involvement of a transcriptional regulatory mechanism in mediating the observed adaptive regulatory increase in RF uptake by RF-deficient conditions in these cells. Further studies, however, are needed to confirm this suggestion. An interesting observation was the ability of extracellular levels of the RF structural analog LF to induce similar adaptive regulatory changes in the RF uptake process. Because LF cannot be phosphorylated or used as a vitamin by mammalian cells (14) and because it appears to share the same transport system as RF [as indicated by results of the present study and previous findings (25, 23)], this finding suggests that it is the availability of a recognizable substrate by the uptake system that is important for the induction of the adaptive regulatory effect in RF uptake by substrate levels. Further studies are needed to delineate the exact molecular mechanism(s) involved in this regulation.

We also investigated possible regulation of the RF uptake process of NCM460 by intracellular regulatory pathways focusing on a role for Ca$^{2+}$/calmodulin and PKC-mediated pathways. Our results suggested a role for a Ca$^{2+}$/calmodulin but not a PKC-mediated pathway in the regulation of colonic uptake of RF. This conclusion is based on the observations that modulators of the Ca$^{2+}$/calmodulin-mediated pathway significantly affect RF uptake. An inhibition in the vitamin uptake was observed in cells pretreated with calmidazolium and TFP. The effect of calmidazolium appears to be mediated via a significant inhibition in the $V_{max}$ of the RF uptake process and an increase in its apparent $K_m$. These findings suggest that the effect is mediated via a decrease in the activity of the RF uptake system and a decrease in its affinity, respectively. The physiological mechanism(s) through which the Ca$^{2+}$/calmodulin-mediated pathway exerts its regulatory effect on RF uptake is not clear. However, different mechanisms of action for this pathway have been advanced, including activation of specific protein kinases and the direct effect on the uptake system involved. Further studies at the molecular level are needed to address this issue. The possible regulation of RF uptake in NCM460 cells by a Ca$^{2+}$/calmodulin-mediated pathway is similar to what has been observed in human-derived renal epithelial HK-2 cells and liver Hep G2 cells (11, 25).

In summary, this is the first report showing the existence of a specialized, carrier-mediated mechanism for RF uptake in the human-derived colon epithelial cell line NCM460. This system appears to be regulated by extracellular RF levels and by an intracellular Ca$^{2+}$/calmodulin-mediated pathway. It is suggested that the identified RF transport mechanism is involved in absorption of the bacterially synthesized RF in the large intestine and that this source of RF may contribute toward RF homeostasis, especially the localized homeostasis of colonocytes.

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