Riboflavin Uptake by the Human-Derived Liver Cells Hep G2: Mechanism and Regulation

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The water-soluble vitamin riboflavin (RF) plays a critical role in many metabolic reactions, and thus, is essential for normal cellular functions and growth. The liver plays a central role in normal RF metabolism and is the site of maximal utilization of the vitamin. The mechanism of liver uptake of RF has been studied in animals, but no information is available describing the mechanism of the vitamin uptake in the human situation and its cellular regulation. In this study, we used the human-derived liver cells Hep G2 as an in vitro model system to address these issues. Uptake of RF by Hep G2 cells was found to be temperature- and energy-dependent but Na^+-independent in nature. Uptake seemed to involve a carrier-mediated process as indicated by the saturation as a function of substrate concentration (apparent K_m 0.41 ± 0.08 μM), and by the ability of the structural analogs lumiflavin and lumichrome to inhibit the uptake process [inhibition constant (K_i) of 1.84 and 6.32 μM, respectively]. RF uptake was energy dependent, and was inhibited by the -SH group blocker p-chloromercuriphenylsulfonate (p-CMPS) (K_i of 0.10 mM). Specific modulators of intracellular protein kinase A (PKA)-, protein kinase C (PKC)-, and protein tyrosine kinase (PTK)-mediated pathways did not affect RF uptake by Hep G2 cells. On the other hand, specific inhibitors of Ca^{2+}/calmodulin-mediated pathway significantly inhibited the uptake process; this effect seemed to be mediated through a decrease in the V_{max} of the substrate uptake process. Maintaining Hep G2 cells in a RF-deficient growth medium was associated with a significant up-regulation in the substrate uptake; this effect was specific for RF and was mediated mainly by means of an increase in the V_{max} of the uptake process. These results describe, for the first time, the mechanism and cellular regulation of RF uptake by a human-derived liver cellular preparation, and shows the involvement of a carrier-mediated system in the uptake process. Furthermore, the uptake process seems to be regulated by an intracellular Ca^{2+}/calmodulin-mediated pathway and by extracellular substrate levels. J. Cell. Physiol. 176:588–594, 1998. © 1998 Wiley-Liss, Inc.

Riboflavin (RF) is a precursor of two coenzymes, flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD) that participate in key metabolic reactions such as carbohydrate, lipid, and amino acid metabolism and the reaction that converts pyridoxine into its coenzyme form (Cooperman and Lopez, 1984; McCormick, 1989). Thus, RF is essential for normal cellular functions and growth. Deficiency of RF occurs in human and leads to a variety of clinical abnormalities, including degenerative changes in the nervous system, endocrine dysfunction, anemia, and skin disorders (Leevy et al., 1965; Rosenthal et al., 1973; Goldsmith, 1975; McCormick, 1989; Kodentsova et al., 1994).

Mammals cannot synthesize RF but obtain the vitamin from exogenous sources. The liver plays a central role in normal RF metabolism and is the major site of RF utilization. The mechanism of RF uptake by the liver has been studied in the rat by Aw et al. (1983) using freshly isolated hepatocytes. The mechanism was found to involve a Na^+-independent, carrier-mediated process. Subsequent studies in our laboratory using purified rat liver basolateral membrane vesicles have confirmed these findings (Said et al., 1995). To date, how-

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ever, there has been no information available about the mechanism of RF liver uptake in the human situation and about its cellular regulation. Performance of such studies using native human liver cellular preparation(s) is difficult because of the limited availability of such samples. Furthermore, freshly isolated liver cells have limited viability in vitro and have been shown to lose some of their transport characteristics under such conditions (Tarentino and Galivan, 1980; Said et al., 1990; Weiner and Wolf, 1990). Moreover, cellular regulation of an uptake process is better studied using a homogenous cellular preparation. The human-derived liver cells Hep G2 represent a homogeneous cell population and have been used as an in vitro model system to study functions of human liver, including cellular uptake processes (Stremmel and Diede, 1989; Said et al., 1994b). In this study, we used these cells as a model to study RF uptake in the human situation. Our results demonstrate that RF uptake by these cells is by means of a carrier-mediated process and that the uptake process seems to be regulated by an intracellular Ca^{2+}/calmodulin-mediated pathway and by extracellular substrate level.

MATERIALS AND METHODS

Riboflavin [^{3}H-(G)] [sp. act. 27.5 Ci/mmol; radiochemical purity (determined by the manufacturer and confirmed by us) was >98%] was purchased from Moravek Biochemicals, Inc. (Brea, CA). Dulbecco's modified Eagle medium, trypsin, fetal bovine serum (FBS), and other cell culture materials were obtained from Irvine Scientific (Santa Ana, CA). Unlabeled RF and all other chemical and reagents were obtained from commercial sources and were of analytical quality.

The human-derived hepatoma cell line Hep G2 was purchased from the American Type Culture Collection (Rockville, MD) and was used for uptake studies between passages 74 and 82. The cells were grown in 75-cm^2 plastic flasks (Costar, Cambridge, MA) in Dulbecco's modified Eagle medium containing 10% FBS, 4.5 g/L glucose, 50 U/ml streptomycin, 100 U/ml penicillin, and 0.25 µg/ml amphotericin B, at 37°C in a 5% CO_2 plus 95% air atmosphere. Media changes were done every 2 days. The cells were subcultured by trypsinization with 0.05% trypsin and 0.9 nM ethylenediaminetetraacetic acid in Ca^{2+}-free and Mg^{2+}-free phosphate buffered saline solution and plated onto 12-well plates at a concentration of 5×10^5 cells/well. Uptake of RF was studied 2 to 4 days after confluence. Cell growth and contamination were observed by periodic monitoring with an inverted microscope. Cell viability (including the viability of cells grown in RF-deficient media) was assessed by trypan blue dye exclusion method and was found to exceed 92%.

In examining the effect of the RF-deficient condition on uptake of ^{3}H-RF by Hep G2 cells, the cells were maintained for 48 hr in RF-deficient media [i.e., a media containing no added RF except that provided by FBS (approximately 0.288 ng RF/ml) before use for uptake studies. The results were compared with uptake by cells incubated in RF-sufficient (control) medium [i.e., a medium containing a total of 0.3767 µg RF/ml of which approximately 0.3764 µg/ml represents added RF and the rest (approximately 0.288 ng RF/ml) was provided by the added FBS].

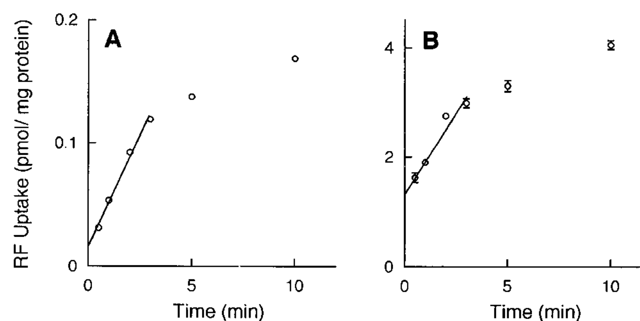

Fig. 1. Uptake of RF by Hep G2 cells as a function of time. Cells were incubated in Krebs-Ringer buffer at 37°C in the presence of (**A**) low (5.5 nM) and (**B**) high (1 µM) concentrations of RF. Results are mean ± SEM of four separate uptake determinations. When not shown, SEM bars are within the symbol size.

Uptake of RF was examined in cells incubated in Krebs-Ringer buffer (in mM: 133 NaCl, 4.93 KCl, 1.23 $MgSO_4$, 0.85 $CaCl_2$, 5 glucose, 5 glutamine, 10 *N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid, and 10 2-N-morpholinoethanesulfonic acid, pH 7.4; unless otherwise specified) at 37°C. Labeled and unlabeled RF were added to the incubation medium at the start of the uptake experiment. In certain experiments, cells were pretreated with the compound under study for 1 hr before the start of the uptake experiment. The uptake reaction was terminated at the end of 3-min incubation (unless otherwise stated) by the addition of 2 ml of ice-cold buffer followed by immediate aspiration. Cells were then rinsed twice with ice-cold buffer and digested with 1 ml of 1 N NaOH, neutralized with HCl, then counted for radioactivity. Protein contents of cell digest were measured on parallel wells using the method of Lowry et al. (1951) with bovine serum albumin used as the standard. Data presented in this paper are mean ± SEM of multiple separate uptake determinations and were expressed in term of picomoles or femtomoles per milligram of protein per unit time. Kinetic parameters of RF uptake (i.e., the apparent K_m and V_{max}) were calculated using a computerized model of the Michaelis-Menten equation as described previously by Wilkinson (1961). As appropriate, data were analyzed by the Student's t test or by analysis of variance (ANOVA) followed by post hoc analysis; comparison was made relative to simultaneously performed controls.

In examining the metabolic form of the ^{3}H radioactivity taken up by Hep G2 cell after incubation with ^{3}H-RF, silica-gel-precoated thin layer chromatography plates, and a solvent system of ethanol and water (9:1; vol/vol) were used as described previously (Fazekos, 1975).

RESULTS

General characteristics of RF uptake by Hep G2 cells

Figure 1 depicts the uptake of low (5.5 nM) and high (1 µM) concentrations of RF as a function of incubation time. At both concentrations, uptake was found to be linear for up to 3 min of incubation but leveled thereafter; thus, a 3-min incubation time was used in all subsequent studies.

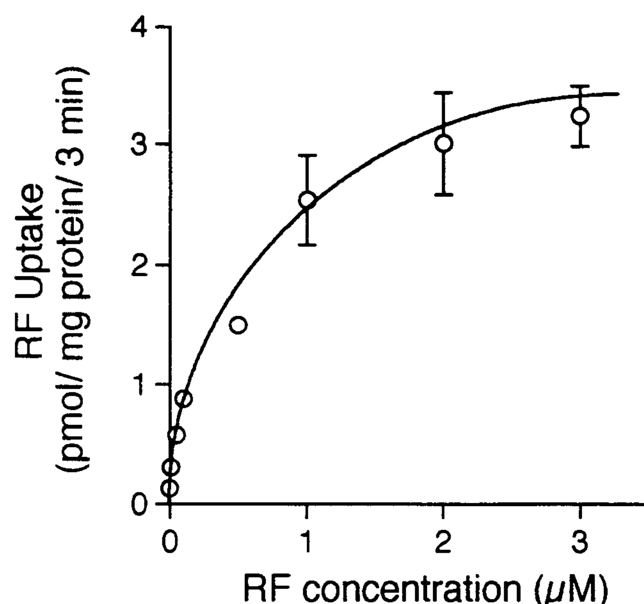

Fig. 2. Initial rate of RF uptake by Hep G2 cells as a function of substrate concentration in the incubation medium. Cells were incubated for 3 min in Krebs-Ringer buffer at 37°C in the presence of different concentrations of RF. Uptake by the saturable component was determined as described in the text. Each data point is mean ± SEM of three to four separate uptake determinations.

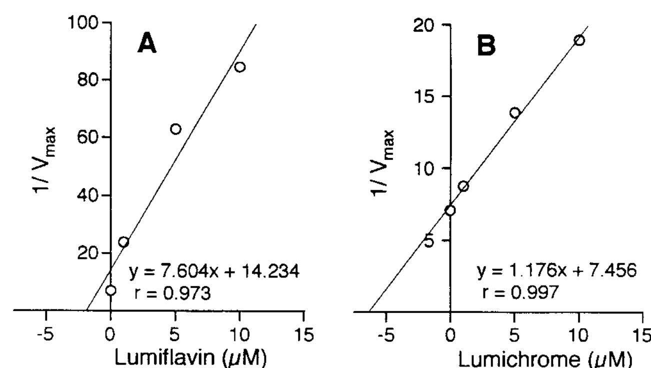

Fig. 3. Dixon plot of the inhibitory effect of lumiflavin and lumichrome on ^{3}H-RF uptake. Cells were incubated for 3 min at 37°C in Na^+ containing Krebs-Ringer buffer in the presence of 5.5 nM ^{3}H-RF and different concentrations of lumiflavin (**A**) and lumichrome (**B**). Results are mean of four separate uptake determinations.

In a separate study, we examined the metabolic form of the ^{3}H-radioactivity taken up by Hep G2 cells after incubation with 13 nM ^{3}H-RF for 3 min at 37°C (see the Materials and Methods section). The result showed that the majority (94.8%) of the incorporated ^{3}H-radioactivity to be in the form of intact RF.

The effect of incubation temperature on the uptake of RF (5.5 nM) was then examined. Significantly ($P < 0.01$) higher uptake was found at 37°C compared with 4°C (0.133 ± 0.016 and 0.030 ± 0.002 pmol/mg protein per 3 min, respectively).

The effect of varying Na^+ and H^+ concentrations in the incubation media on the uptake of RF was also tested. Na^+ effect was tested by examining the effect of replacing the cation in the incubation medium isosmotically with Li^+, K^+, choline, NH_4^+, Tris, or with mannitol on the uptake of RF (5.5 nM). There was no significant change in RF uptake whether or not Na^+ was present in the incubation medium [0.125 ± 0.004, 0.117 ± 0.005, 0.120 ± 0.003, 0.117 ± 0.005, 0.118 ± 0.008, 0.130 ± 0.004, and 0.125 ± 0.003 pmol/mg protein per 3 min for control (Na^+) and in the absence of Na^+ and presence of K^+, choline, Li^+, NH_4^+, Tris, and mannitol, respectively]. In examining the effect of H^+ on RF uptake, the pH of the incubation medium was varied over the range of 5.0 and 8.0. The results showed that lowering the incubation pH from 8.0 to 5.0 was associated with a gradual decrease in RF (5.5 nM) uptake [0.139 ± 0.003, 0.122 ± 0.002, 0.116 ± 0.007, 0.094 ± 0.002, 0.083 ± 0.003, 0.075 ± 0.001, and 0.066 ± 0.002 pmol/mg protein per 3 min at buffer pH 8.0, 7.4, 7.0, 6.5, 6.0, 5.5, and 5.0, respectively].

Uptake of RF as a function of concentration

Figure 2 shows the initial rate of RF uptake by Hep G2 cells as a function of increasing the substrate concentration in the incubation media. Incubation was performed for 3 min at 37°C. Uptake of RF over the concentration range of 0.033 to 3 μM was found to include a saturable component. Uptake by this component was determined by subtracting uptake by diffusion [calculated from the slope of the line between uptake at high pharmacologic concentration of 100 μM and the point of origin (slope was 3.26)] from total uptake. Kinetic parameters of the saturable component, i.e., the apparent K_m and V_{max}, were then calculated as described in the Materials and Methods section and found to be 0.41 ± 0.08 μM and 3.56 ± 0.24 pmol/mg protein per 3 min, respectively.

Effect of RF structural analogs and related compounds on uptake of ^{3}H-RF by Hep G2 cells

The effect of different concentrations of the structural analogs lumiflavin and lumichrome, the related compound lumazine (1 mM), and the unrelated compound biotin (1 mM) on the uptake of 5.5 nM ^{3}H-RF was examined. Incubation was performed for 3 min in a Na^+-containing buffer at 37°C. Both lumiflavin and lumichrome caused a concentration-dependent inhibition in ^{3}H-RF uptake; the inhibition constant (K_i) was calculated by the Dixon method and was found to be 1.84 and 6.32 μM, respectively (Fig. 3). On the other hand, neither lumazine nor biotin had a significant effect on ^{3}H-RF uptake (0.137 ± 0.002, 0.137 ± 0.004, and 0.135 ± 0.0014 pmol/mg protein per 3 min for control and in the presence of lumazine and biotin, respectively).

In a separate study, we also examined the effect of 10 μM lumiflavin and lumichrome on the uptake of 5.5 nM ^{3}H-RF by Hep G2 cells incubated in Na^+-free media (Na^+ was replaced isosmotically with Li^+). Again both compounds caused a significant ($P < 0.01$ for both by ANOVA followed by post hoc analysis) inhibition in RF uptake (0.123 ± 0.001, 0.014 ± 0.002, and 0.083 ± 0.001 pmol/mg protein per 3 min for control and in the presence of lumiflavin and lumichrome, respectively).

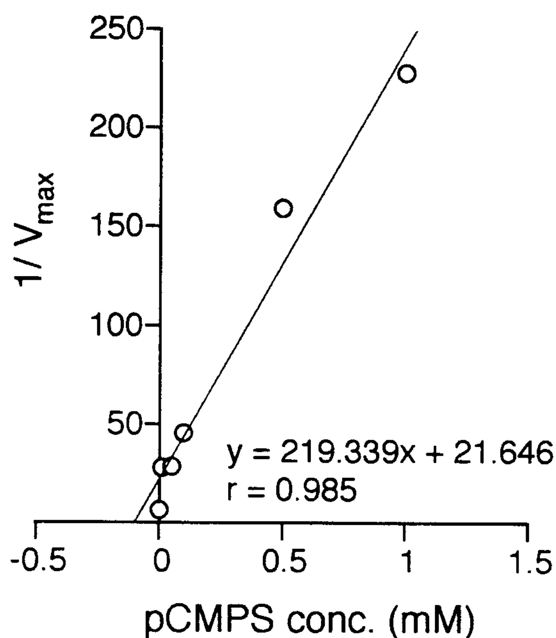

Fig. 4. Dixon plot of the inhibitory effect of pCMPS on ^{3}H-RF uptake. Cells were preincubated first with pCMPS for 30 min, then incubated for 3 min in Na^+-containing Krebs-Ringer buffer at 37°C in the presence of 5.5 nM ^{3}H-RF. Results are mean of three to four separate uptake determinations.

Effect of metabolic and -SH group inhibitors on RF uptake by Hep G2 cells

In this study, we examined the effect of pretreating Hep G2 cells for 30 min with the metabolic inhibitors dinitrophenol (10 mM), iodoacetate (10 mM), azide (1 mM), and ouabain (10 mM) on the uptake of 5.5 nM ^{3}H-RF. With the exception of ouabain, all other inhibitors caused significant ($P < 0.01$ for all by ANOVA followed by post hoc analysis) inhibition in RF uptake (0.151 ± 0.005, 0.112 ± 0.004, 0.066 ± 0.005, 0.121 ± 0.004, and 0.150 ± 0.005 pmol/mg protein per 3 min for control and upon pretreatment with dinitrophenol, iodoacetate, azide, and ouabain, respectively).

In another study, we examined the effect of the -SH group blocking reagent p-cholomercuriphenylsulfonate (p-CMPS) on the uptake of ^{3}H-RF (5.5 nM) by Hep G2 cells. The results showed that pretreating the cells with p-CMPS causes a significant inhibition in RF uptake with a K_i value of 0.10 mM (Fig. 4). The inhibitory effect of 0.5 mM p-CMPS was completely reversed upon treating p-CMPS pretreated cells with the reducing agent 2-mercaptoethanol (10 mM) [0.130 ± 0.005, 0.037 ± 0.001 ($P < 0.01$), 0.131 ± 0.006 and 0.130 ± 0.050 pmol/mg protein per 3 min for control, p-CMPS-pretreated cells, 2-mercaptoethanol treated cells, and cells pretreated with p-CMPS then with 2-mercaptoethanol, respectively].

Regulation of RF uptake by Hep G2 cells

Regulation by intracellular signal transduction pathways. In this study we examined the role of specific intracellular signal transduction pathways in the regulation of RF uptake by Hep G2 cells. Specifically, we examined the effect of modulating the activity of protein kinase-A (PKA), protein kinase-C (PKC), and protein tyrosine kinase (PTK) as well as Ca^{2+}/calmodulin-mediated pathways on the uptake of ^{3}H-RF. We focused on these pathways, because transport of a variety of substrates in other cellular systems have been shown to be regulated by these pathways (Cohen et al., 1990; de Jong and Rao, 1990; Fliegel et al., 1992; Brandsh et al., 1993; Donowitz et al., 1993; Piper et al., 1993; Said and Ma, 1994). Specific modulators of these pathways were used in the study.

TABLE 1. Effect of compounds that elevate intracellular cAMP levels on the uptake of RF by Hep G2 cells[1]

Compound	Concentration	Uptake (pmol/mg protein/3 min)
Control		0.136 ± 0.005
Dibutryl cAMP	0.5 mM	0.145 ± 0.004
	1.0 mM	0.135 ± 0.005
Forskolin	250 μM	0.143 ± 0.004
Cholera toxin	0.5 μg/ml	0.140 ± 0.006
	2.0 μg/ml	0.139 ± 0.007
	5.0 μg/ml	0.143 ± 0.004

[1]Cells were pretreated for 1 hr with the compound under investigation; ^{3}H-RF (5.5 nM) was then added and the incubation continued for 3 min at 37°C. Results are mean ± SEM of four separate uptake determinations.

The role of PKA-mediated pathway was investigated by examining the effect of compounds that increase intracellular cAMP level on RF uptake. This was done by pretreating Hep G2 cells (for 1 hr) with different concentrations of the membrane permeable dibutyl cAMP, cholera toxin, and forskolin then examining the uptake of ^{3}H-RF (5.5 nM). Pretreatment with these compounds did not cause significant alterations in RF uptake (Table 1).

In another study, we investigated the role of PKC-mediated pathway in the regulation of RF uptake. This was done by examining the effect of pretreating Hep G2 cells (for 1 hr) with the PKC activators phorbol-12-myristate-13-acetate and dioctanoyl glycerol and with the PKC inhibitors chelerythrin and staurosporin on the uptake of 5.5 nM ^{3}H-RF. None of the modulators of PKC activity significantly affected RF uptake (Table 2).

The possible role of PTK-mediated pathway in the regulation of RF uptake was tested by examining the effect of pretreating Hep G2 cells (for 1 hr) with the PTK specific inhibitors genistein and tyrophostin A1 on the uptake of 5.5 nM ^{3}H-RF (genistein and tyrophostin A25 served as negative controls, respectively). The results (Table 3) showed that both genistein and tyrophostin A1 caused a slight inhibition in RF uptake; however, this limited inhibition was also observed in cells pretreated with their negative controls genistein and tyrophostin A-25. This finding indicates that the effect of genistein and tyrophostin A1 on RF uptake is non-specific in nature. We also examined the effect of pretreating the cells (1 hr) with the protein tyrosine phosphatase inhibitor vanadate on the uptake of ^{3}H-RF (5.5 nM). Again, no effect was observed (Table 3).

The role of Ca^{2+}/calmodulin-mediated pathway in the regulation of RF uptake by Hep G2 cells was investigated by examining the effect of pretreating the cells with the Ca^{2+}/calmodulin specific inhibitors calmidazolium, trifluoperazine, and KN62 [1-(N,O-bis[5-isoquinoline sulfonyl]-*N*-methyl-l-tyrosyl)-4-phenylpiper-

TABLE 2. Effect of modulators of PKC on the uptake of RF by Hep G2 cells[1]

Compound	Concentration (μM)	Uptake (pmol/mg protein/3 min)
Control		0.154 ± 0.002
PMA	1	0.138 ± 0.001
	10	0.154 ± 0.001
Dioctanoyl glycerol	1	0.147 ± 0.001
	10	0.153 ± 0.002
Chelerythrin	1	0.148 ± 0.002
	10	0.144 ± 0.004
Staurosporin	10	0.153 ± 0.002

[1] Legend is as in Table 1.

TABLE 3. Effect of inhibitors of PTK on the uptake of RF by Hep G2 cells[1]

Compound	Concentration (μM)	Uptake (pmol/mg protein/3 min)
A		
Control		0.139 ± 0.013
Genistein	50	0.123 ± 0.005
Genistin	50	0.127 ± 0.007
Tyrophostin A-1	50	0.110 ± 0.009
Tyrophostin A-25	50	0.103 ± 0.008
B		
Control		0.138 ± 0.010
Vanadate	50	0.142 ± 0.010
	100	0.137 ± 0.009

[1]Legend is as in Table 1. Results are mean ± SEM of six to seven separate uptake determinations.

azine] on the uptake of 5.5 nM ^{3}H-RF. The results (Table 4) showed that all compounds tested caused a significant ($P < 0.01$) inhibition in RF uptake. Uptake of the unrelated folic acid (6 nM), on the other hand, was not affected by pretreatment with any of the tested compounds [3.8 ± 0.4, 3.9 ± 0.5, 3.8 ± 0.2, and 3.2 ± 0.1 fmol/mg protein per 3 min for control and calmidazolium (50 μM), trifluoperazine (100 μM) and KN62 (50 μM) pretreated cells, respectively]. In another experiment, we investigated whether the inhibitory effect of calmidazolium was mediated through an effect on the apparent K_m and/or the V_{max} of the RF uptake process by Hep G2 cells. This was done by examining the effect of the inhibitor on the uptake of RF as a function of concentration. The results (Figure 5) showed that the effect of calmidazolium was mediated through a significant ($P < 0.01$) effect on the V_{max} (not the apparent K_m) of the RF uptake process (3.93 ± 0.57 and 2.17 ± 0.07 pmol/mg protein per 3 min and 0.54 ± 0.32 and 0.30 ± 0.04 μM for control and calmidazolium pretreated cells, respectively).

Adaptive regulation by substrate level. In this study, we examined the uptake of ^{3}H-RF (5.5 nM) by cells grown for 48 hr in RF-deficient medium and compared the results with uptake by cells grown in a control (i.e., RF-sufficient) medium. The cells were incubated for 3 min at 37°C. Uptake of the unrelated ^{3}H-biotin (3.2 nM) was also examined in these cells. The results showed that growing cells in a RF-deficient medium leads to a significant ($P < 0.01$) increase in the uptake of ^{3}H-RF compared with cells grown in control media (0.200 ± 0.007 and 0.137 ± 0.003 pmol/mg protein per 3 min, respectively). On the other hand, uptake of the unrelated biotin was similar under the two growth conditions (0.046 ± 0.001 and 0.040 ± 0.001 pmol/mg protein per 3 min for cells grown in control and RF-deficient media, respectively). In another study, we examined the effect of RF-deficient media on the kinetic parameters of RF uptake by Hep G2 cells. This was done by examining ^{3}H-RF uptake by cells grown for 48 hr in control and RF-deficient media as a function of concentration and determining the kinetic parameters of the uptake process as described earlier. The results (Figure 6) showed the V_{max} of RF uptake process was significantly ($P < 0.01$) higher in cells grown in RF-deficient media compared with the cells grown in control medium (6.08 ± 0.14 and 3.37 ± 0.32 pmol/mg protein per 3 min for control cells and cells grown in RF-deficient medium, respectively). On the other hand, no significant change in the apparent K_m of the uptake system was observed (0.52 ± 0.05 and 0.45 ± 0.09 μM, respectively).

DISCUSSION

The present study examined the mechanism and cellular regulation of the uptake process of RF by the human liver using the human-derived Hep G2 liver cells as an in vitro model system. The results showed the uptake process of RF by these cells to be temperature dependent and to occur with minimal metabolic alteration in the transported substrate. Uptake was independent of Na^+ presence in the incubation medium as indicated by lack of inhibition in the substrate uptake upon replacing Na^+ with other monovalent cations or with mannitol. The inability of the Na^+-K^+ ATPase inhibitor ouabain to inhibit RF uptake further supports the Na^+-independent nature of the RF uptake process. This conclusion is in line with previous findings with freshly isolated rat hepatocytes (Aw et al., 1983) and purified rat liver basolateral membrane vesicles (Said et al., 1995) as well as with the findings in mammalian small intestine (Said and Ma, 1994) and *Xenopus laevis* oocytes (Dyer and Said, 1995), in which RF uptake was also found to be Na^+-independent in nature. Furthermore, uptake of RF was found to be higher at the physiologic range of pH than at acid pH as shown by the decrease in the substrate uptake as a function of decreasing the incubation buffer pH toward pH 5. The mechanism by which buffer pH affects RF uptake is not clear but cannot be attributed to a nonspecific action of pH on Hep G2 cells because previous studies in our laboratory have shown that similar changes in buffer pH had no effect on the uptake of the unrelated biotin by these cells (Said et al., 1994b). Further studies are needed to clarify this issue.

A carrier-mediated system seemed to be involved in RF uptake by Hep G2 cells. This conclusion was based on the observations that uptake of RF was saturable as a function of substrate concentration (apparent K_m = 0.54 ± 0.09 μM) and was inhibited by structural analogs. The K_i of the structural analog lumiflavin (1.34 μM) was found to be closer to the apparent K_m of the RF uptake process than the K_i of the structural analog lumichrome (6.32 μM). This suggests that replacing the ribityl side chain at nitrogen 10 of the isoalloxazine ring by a smaller group (i.e., -CH_3) does not markedly affect the ability of the new compound (i.e.,

TABLE 4. Effect of inhibitors of Ca^{2+}/calmodulin-mediated pathway on uptake of RF by Hep G2 cells[1]

Compound	Concentration (µM)	Uptake (pmol/mg protein/3 min)	*P* value
A			
Control		0.135 ± 0.003 (9)	
Calmidazolium	10	0.119 ± 0.002 (7)	<0.05
	50	0.084 ± 0.007 (10)	<0.01
KN62	50	0.110 ± 0.005 (7)	<0.01
B			
Control		0.155 ± 0.005 (7)	
Triflouperazine	50	0.133 ± 0.003 (5)	<0.01
	100	0.075 ± 0.002 (7)	<0.01

[1]Legend is as in Table 1. Results are mean ± SEM of 5 to 10 separate uptake determinations. Analysis of variance was applied to the data. *P* values were derived using post hoc analysis by comparing each treatment with control.

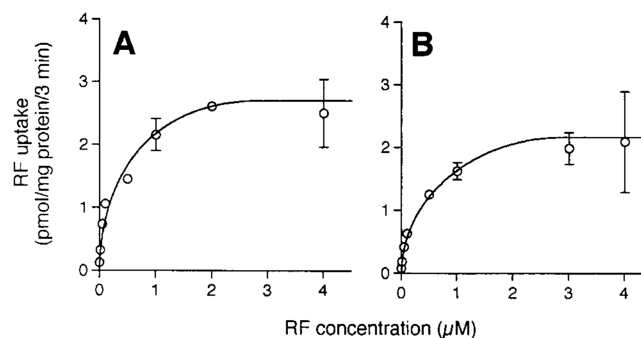

Fig. 5. Effect of calmidazolium on initial rate of RF uptake by Hep G2 cells as a function of substrate concentration in the incubation medium. Cells were pretreated for 1 hr with buffer (**A**) or calmidazolium (**B**; 50 µM); incubation was then continued for 3 min in Krebs-Ringer buffer at 37°C in the presence of different concentrations of RF. Uptake by the saturable component was determined as described in the text. Each data point is mean ± SEM of three to four separate uptake determinations. When not shown, SEM bars are within the symbol size.

lumiflavin) to interact with the RF uptake carrier. On the other hand, total removal of the side chain or a group from position 10 of the isoalloxazine ring may lead to a decrease in the ability of the new compound (i.e., lumichrome) to interact with the RF uptake carrier. The RF uptake process was also found to be energy-dependent in nature as indicated by the significant inhibition in the substrate uptake by metabolic inhibitors. Our results on RF uptake by the human-derived Hep G2 cells of temperature and energy dependence, Na independence, and carrier mediation are similar to those reported previously with animal liver preparations (Aw et al., 1983; Said et al., 1995).

Our results also indicate that a sulfhydryl group(s) was essential for the function of the RF uptake system. This suggestion was based on the observation that the -SH group inhibiting reagent p-CMPS significantly inhibited the uptake of RF. p-CMPS seemed to interact specifically with -SH groups because its inhibitory effect of RF uptake was completely reversed by treating pCMPS-pretreated cells with the reducing agent 2-mercaptoethanol.

Having identified the characteristics of the mechanism of RF uptake by Hep G2 cells, we then used these cells to investigate the cellular regulation of the uptake process. We focused our studies on the possible role of specific intracellular signal transduction pathways, and on substrate level in the growth medium because transport of variety of substrates are known to be regulated by these pathways and conditions (Said et al., 1989; Ferraris and Diamond, 1989; Cohen et al., 1990; de Jong and Rao, 1990; Fliegel et al., 1992; Brandsh et al., 1993; Donowitz et al., 1993; Piper et al., 1993; Said and Ma, 1994). Our results showed that the PKA-mediated pathway has no role in regulating RF uptake. This conclusion is based on the observations that compounds that increase intracellular cAMP levels had no effect on RF uptake. Similarly, PKC- and PTK-mediated pathways seemed to have no role in regulating RF uptake by Hep G2 cells. This conclusion again was based on the observations that specific modulators of these cellular pathways failed to affect RF uptake to any significant extent. In contrast, a Ca^{2+}/calmodulin-mediated pathway(s) seemed to play a role in regulating RF uptake in these cells. This conclusion is based on the observations that RF uptake was significantly inhibited by specific inhibitors of this pathway. The effect of these inhibitors on RF uptake seemed to be specific in nature, because uptake of the unrelated folic acid was not affected by pretreatment of cells with any of these compounds. Furthermore, the inhibitory effect of calmidazolium on RF uptake was found to be mediated through a significant decrease in the V_{max} of the uptake process with no change in the apparent K_m. These findings suggested that the effect was mediated by means of a decrease in the activity and/or the number of the carriers with no change in the affinity of the carrier, respectively. The physiologic mechanism(s) through which Ca^{2+}/calmodulin-mediated pathway exerts its regulatory effect on RF uptake is not known. However, different mechanisms of action for this pathway have been advanced, including activation of specific protein kinases and direct effect on the carrier involved. Further studies at the molecular level are needed to address this issue.

The lack of involvement of a PKA-mediated pathway in the regulation of RF uptake by the human-derived Hep G2 cells is in contrast to our finding on RF uptake by the human-derived Caco-2 intestinal epithelial cells. In Caco-2 cells, a PKA-mediated pathway was found to be involved in regulating the substrate uptake event (Said et al., 1994a). On the other hand, the regulation of RF uptake in Hep G2 cells by a Ca^{2+}/calmodulin-mediated pathway was similar to our recent findings on the regulation of the uptake process in the human-derived renal epithelial cells, HK-2 (unpublished observation). These findings demonstrate that the transport process of this essential nutrient is regulated by different mechanisms in different tissues.

In examining the effect of RF concentration in the growth medium on the substrate uptake, our results showed that uptake of the vitamin by cells grown in RF-deficient medium to be significantly higher than uptake by cells grown in control (i.e., RF-sufficient) medium. This effect was found to be specific for RF, as uptake of the unrelated biotin was found to be similar under the two growth conditions. This adaptive regulatory effect by substrate level on RF uptake was found to be mediated mainly through a significant increase in the V_{max} of the uptake process with no change in

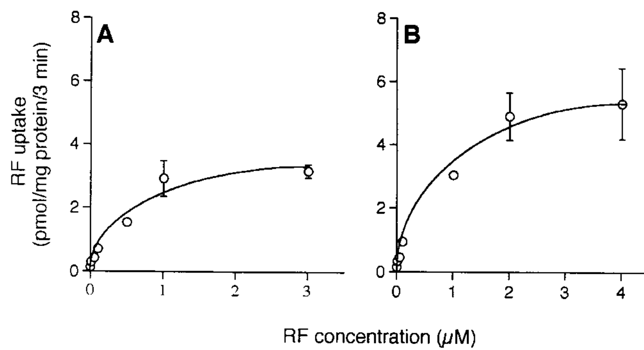

Fig. 6. Effect of growing Hep G2 cells in RF-deficient media on initial rate of ^{3}H-RF uptake as a function of concentration. Cells were grown for 48 hr in (**A**) control (i.e., RF-sufficient) or (**B**) RF-deficient media before use in uptake study. Uptake was assayed by incubating cells for 3 min in Krebs-Ringer buffer at 37°C in the presence of different concentrations of RF. Uptake by the saturable component was determined as described in the text. Each data point is mean ± SEM of three to four separate uptake determinations. When not shown, SEM bars are within the symbol size.

the apparent K_m. These results suggest that the RF-deficient condition was associated with an increase in the activity and/or number of the RF uptake carriers with no change in the affinity of the carrier, respectively. Further studies are needed to delineate the exact molecular mechanism involved.

In summary, these studies demonstrate that RF uptake by the human-derived Hep G2 cells is by means of a Na^+-independent, temperature- and energy-dependent, carrier-mediated system. This system seems to be regulated by an intracellular Ca^{2+}/calmodulin-mediated transduction pathway and by substrate level in the growth medium.

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