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Mechanism of riboflavin uptake by cultured human retinal pigment epithelial ARPE-19 cells: possible regulation by an intracellular Ca^{2+}–calmodulin-mediated pathway

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In mammalian cells (including those of the ocular system), the water-soluble vitamin B_2 (riboflavin, RF) assumes an essential role in a variety of metabolic reactions and is critical for normal cellular functions, growth and development. Cells of the human retinal pigment epithelium (hRPE) play an important role in providing a sufficient supply of RF to the retina, but nothing is known about the mechanism of the vitamin uptake by these cells and its regulation. Our aim in the present study was to address this issue using the hRPE ARPE-19 cells as the retinal epithelial model. Our results show RF uptake in the hRPE to be: (1) energy and temperature dependent and occurring without metabolic alteration in the transported substrate, (2) pH but not Na^+ dependent, (3) saturable as a function of concentration with an apparent K_m of 80 ± 14 nM, (4) *trans*-stimulated by unlabelled RF and its structural analogue lumiflavine, (5) *cis*-inhibited by the RF structural analogues lumiflavine and lumichrome but not by unrelated compounds, and (6) inhibited by the anion transport inhibitors 4,4′-diisothiocyanatostilbene-2,2′-disulphonic acid (DIDS) and 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid (SITS) as well as by the Na^+–H^+ exchange inhibitor amiloride and the sulfhydryl group inhibitor *p*-chloromercuriphenylsulphonate (*p*-CMPS). Maintaining the hRPE cells in a RF-deficient medium led to a specific and significant up-regulation in RF uptake which was mediated via changes in the number and affinity of the RF uptake carriers. While modulating the activities of intracellular protein kinase A (PKA)-, protein kinase C (PKC)-, protein tyrosine kinase (PTK)-, and nitric oxide (NO)-mediated pathways were found to have no role in regulating RF uptake, a role for the Ca^{2+}–calmodulin-mediated pathway was observed. These studies demonstrate for the first time the involvement of a specialized carrier-mediated mechanism for RF uptake by hRPE cells and show that the process is adaptively regulated in RF deficiency, and also appears to be under the regulation of an intracellular Ca^{2+}–calmodulin-mediated pathway.

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The water-soluble vitamin B_2 (riboflavin, RF) plays an essential role in a variety of metabolic reactions that are important for normal cellular functions and development as well as in maintaining growth (Merrill *et al.* 1981; Cooperman & Lopez, 1984). Specifically, RF, in its coenzyme forms riboflavin-5-phosphate (FMN) and flavin adenosine dinucleotide (FAD), plays a key metabolic role as an intermediary in the transfer of electrons in biological oxidation–reduction reactions. These reactions include carbohydrate, lipid and amino acid metabolism, and conversion of vitamin B_6 compounds and that of folic acid into their active forms. Thus, it is not surprising that RF deficiency leads to a number of clinical abnormalities that affect a variety of tissue systems including the nervous, endocrine and ocular systems (Goldsmith, 1975; Cooperman & Lopez, 1984; Blot *et al.* 1993). RF plays a crucial role in a number of important functions of the ocular system including maintenance of the normal structure and function of the ocular surface (Takami *et al.* 2004), functioning of the retinal photoreceptors (Batey *et al.* 1992; Miyamota & Sancar, 1998), and in the protection against nuclear cataract (Cumming *et al.* 2000).

Vertebrate cells cannot synthesize RF and therefore they must obtain the vitamin from the surrounding

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environment via uptake across the cell membrane. This includes human retinal cells, which are among the most metabolically active cells in the body (Rao *et al.* 1999). The human retinal pigment epithelial cells (hRPE cells), which separate the outer retina from its choroidal blood circulation, play a central role in supplying RF (and other nutrients) to the retina (Pow, 2001). To accomplish this important function, the hRPE cells have developed a variety of specialized carrier-mediated uptake mechanisms that includes transporters for amino acids, glucose and vitamins (Chancy *et al.* 2000; Pow, 2001; Busik *et al.* 2002). Nothing is currently known about how these cells take up RF and whether or not they possess a specialized mechanism as has been observed with other epithelial cell types (Said & Ma, 1994; Kumar *et al.* 1998; Said *et al.* 2000). Delineating the transport mechanism involved in hRPE uptake of RF is of physiological and nutritional importance since RF plays a crucial role in the function and the maintenance of the high metabolically active retinal/ocular cells and deficiency of this essential micronutrient has a significant negative impact on the functioning of this organ system (Batey *et al.* 1992; Blot *et al.* 1993; Miyamota & Sancar, 1998; Takami *et al.* 2004). Thus, our aim in the present study was to elucidate the mechanism involved in hRPE uptake of RF using the human cultured retinal pigment epithelial ARPE-19 cells as model. These cells have been used extensively in a variety of physiological investigations, including uptake studies, with findings similar to those obtained with native RPE cells (Aukunuru *et al.* 2001; Busik *et al.* 2002). Our results show for the first time the involvement of a specialized, high-affinity carrier-mediated mechanism for RF uptake by hRPE cells. This system is pH- (but not Na^+-) dependent and appears to be under the regulation of an intracellular Ca^{2+}–calmodulin-mediated pathway.

Methods

Radiolabelled [G-^{3}H]riboflavin (^{3}H-RF; specific activity 41 Ci mmol^{-1}; radiochemical purity greater than 98%, determined by the manufacturer and confirmed by the authors) was obtained from Moravek Biochemicals, Inc. (Brea, CA, USA). Unlabelled RF and all other chemicals and reagents were purchased from commercial sources and were of analytical quality. Fetal bovine serum (FBS) was from Omega Scientific, Inc. (Tarzana, CA, USA). Dulbecco's modified Eagle's medium (DMEM) and trypsin were from Sigma-Aldrich Corp. (St Louis, MO, USA). The human retinal pigment epithelial ARPE-19 cell line was obtained from the American Type Culture Collection (Rockville, MD, USA) and was used for uptake studies between passages 11 and 27.

The hRPE cells were grown and used for uptake studies as has been described previously by other workers (Huang *et al.* 1997; Aukunuru *et al.* 2001; Busik *et al.* 2002). Briefly, cells were grown in 75 cm^2 plastic flasks (Costar) in DMEM containing 4500 mg l^{-1} glucose, 110 mg l^{-1} sodium pyruvate, 10% FBS, 100 U ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin, at 37°C in a 5% CO_2 plus 95% air atmosphere. Media changes were done at intervals of 3–4 days. The cells were subcultured by trypsinization with 0.05% porcine trypsin and 0.02% EDTA.4Na in phosphate-buffered saline solution without Ca^{2+} and Mg^{2+} and plated onto 24-well plates at a concentration of 3×10^5 cells per well. Uptake of RF was analysed 5–7 days after cell confluence. Cell growth and contamination were monitored periodically with an inverted microscope. Cell viability, including the viability of cells grown in RF-deficient media, was assessed with the trypan blue dye exclusion method and was found to exceed 94%.

For examining the effect of RF-deficient conditions on the uptake of ^{3}H-RF by hRPE cells, the cells were maintained for 24 h in RF-deficient growth media which lacked added RF (but contained 5% FBS). This amount of FBS provides approximately 0.4 nM RF. The results were compared to uptake by cells incubated in RF-sufficient (control) growth medium (which contained a total of 1.1 μM RF, of which 0.4 nM was provided by the 5% FBS and the rest from added RF).

Uptake of RF was examined in cells incubated at 37°C in Krebs-Ringer buffer containing 133 mM NaCl, 4.93 mM KCl, 1.23 mM $MgSO_4$, 0.85 mM $CaCl_2$, 5 mM glucose, 5 mM glutamine, 10 mM Hepes, and 10 mM Mes, pH 7.4 (unless otherwise specified). Labelled and unlabelled RF was added to the incubation medium at the beginning of the uptake experiments. In some instances, cells were pretreated with various compounds for 1 h (or 0.5 h) prior to the beginning of the uptake experiments. The uptake reaction was terminated by the addition of 2 ml ice-cold Krebs-Ringer buffer, followed by immediate aspiration and washing with ice-cold Krebs-Ringer buffer. The cells were then digested with 1 N NaOH and kept in a 76°C oven, and then neutralized with HCl. The digested cells were then counted for radioactivity in a beta-scintillation counter. Protein contents of the digested cells were measured in parallel wells using a DC protein assay from Bio-Rad (Hercules, CA, USA), which is similar to the Lowry assay (Lowry *et al.* 1951).

For examining the metabolic form of the ^{3}H radioactivity taken up by hRPE cells after incubation with 40 nM ^{3}H-RF, the cells were homogenized in 100% ethanol and spun, and the supernatant was removed and applied to a silica-gel-precoated thin-layer chromatography (TLC) plate and run with a solvent system of ethanol and water (9 : 1; v/v) as we have described previously (Kumar *et al.* 1998).

Statistical analysis

Data presented in this paper are expressed as the mean ± S.E.M. of multiple separate uptake determinations

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and were expressed as picomoles or femtomoles per milligram of protein per unit time. Statistical differences were analysed by Student's *t* test and ANOVA, with statistical significance set at 0.05 ($P < 0.05$). Kinetic parameters of the saturable RF uptake process (i.e. the apparent Michaelis-Menten constant (K_m) and the maximal velocity (V_{max})) were calculated using a computerized model of the Michaelis-Menten equation as described by Wilkinson (1961). Some variations in the absolute amount of RF taken up by different batches of cells were observed; for this reason, simultaneous controls were run with each set of experiments to allow a proper comparison.

Results

Uptake of RF by hRPE cells: general characteristics of the uptake process

Uptake of RF (8 nM and 1 μM) by hRPE as a function of time is depicted in Fig. 1. Uptake was linear ($r = 0.99$ for both) with time for up to 10 min of incubation and occurred at a rate of 51.6 and 318 fmol (mg protein)$^{-1}$, respectively. A 4 min incubation time was selected to represent the initial rate of uptake and was used as a standard incubation time in all subsequent studies.

The metabolic form of the transported radioactivity following a 4 and 10 min incubation of the hRPE cells with 40 nM ^{3}H-RF was also investigated using a TLC procedure (see Methods). The results showed 98 and 97%, respectively, of the transported ^{3}H radioactivity into the cells to be in the form of intact RF.

The energy dependence of the RF uptake process by hRPE cells was also examined by testing the effect of pretreating the cells (for 30 min) with the metabolic inhibitors dinitrophenol (5 mM), and iodoacetate (5 mM) on the initial rate of uptake of 8 nM RF. The results showed that both of the tested compounds caused a significant ($P < 0.01$) inhibition in RF uptake (335.5 ± 9.3, 166.0 ± 2.1 and 47.9 ± 2.6 fmol (mg protein)$^{-1}$ (4 min)$^{-1}$ for control and in the presence of dinitrophenol and iodoacetate, respectively). We also examined the temperature dependence of the RF uptake process by hRPE cells. The initial rate of uptake of RF (8 nM) was found to be significantly ($P < 0.01$) higher at 37°C compared to lower incubation temperatures (309.3 ± 5.8, 137.4 ± 2.4 and 63.1 ± 1.0 fmol (mg protein)$^{-1}$ (4 min)$^{-1}$, at 37, 21 and 4°C, respectively).

In separate studies, the effect of varying the incubation buffer pH (i.e. changing the H^+ ion concentration) on the initial rate of RF uptake by hRPE cells was examined. The results showed RF (8 nM) uptake to be highest at pH 7.5 and above, but uptake decreased as the buffer pH became more acidic with lowest uptake being observed at buffer pH 5.0 (Fig. 2).

The role of extracellular Na^+ in RF uptake by hRPE cells was also tested by examining the effect of replacing Na^+ in the incubation buffer with an equimolar concentration of other monovalent cations (K^+, choline and Li^+), or with the inert mannitol. The results showed the initial rate of RF (8 nM) uptake to be similar in the presence

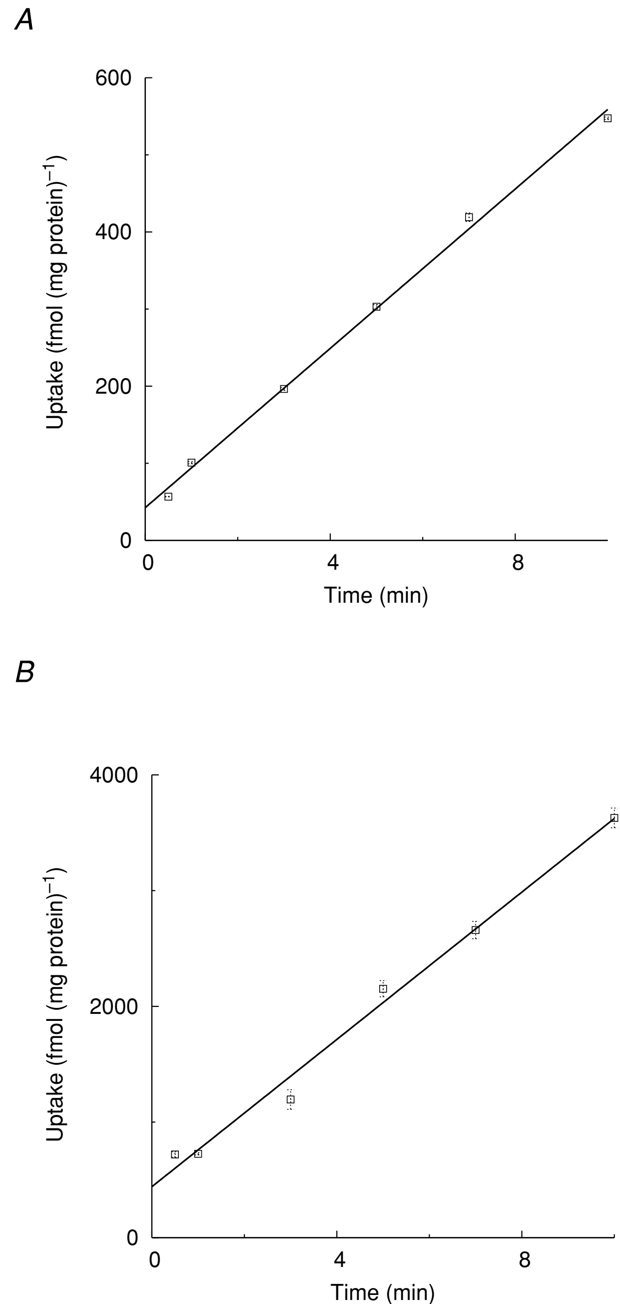

Figure 1. Uptake of riboflavin (RF) by hRPE cells as a function of time

Confluent monolayers of hRPE were incubated in Krebs-Ringer buffer pH 7.4, at 37°C in the presence of 8 nM (*A*) and 1 μM (*B*) RF. Data are mean ± S.E.M. of 3–4 separate uptake determinations. When not shown, the S.E.M. values are smaller than the symbol.
A, $y = 51.646x + 42.464$, $r = 0.998$; *B*, $y = 318x + 441$, $r = 0.995$.

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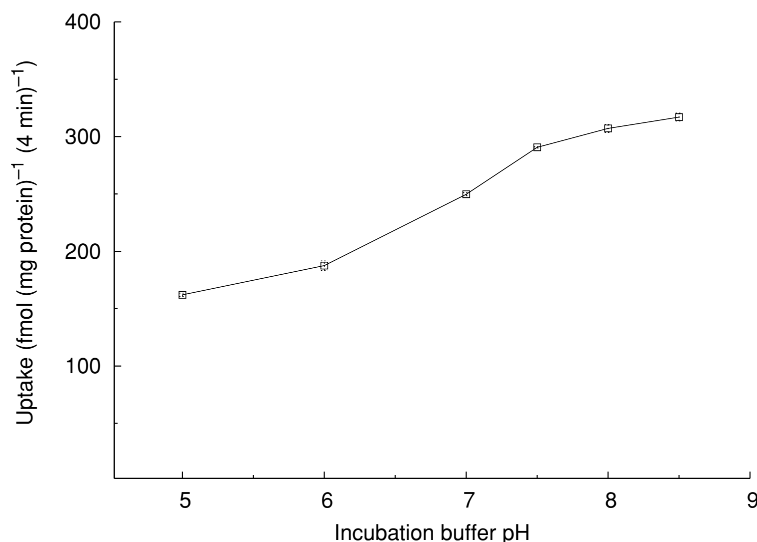

Figure 2. Effect of incubation buffer pH on the initial rate of RF uptake by hRPE cells
Confluent monolayers of hRPE were incubated at 37°C in Krebs-Ringer buffer of varying pH for 4 min in the presence of 8 nM RF. Values are mean ± S.E.M. of 3–4 separate uptake determinations. When not shown, the S.E.M. values are smaller than the symbol.

and absence of Na^+ and regardless of its replacement (297.5 ± 6.0, 294.4 ± 8.1, 280.5 ± 6.6, 283.4 ± 11.4 and 285.4 ± 15.3 fmol (mg protein)$^{-1}$ (4 min)$^{-1}$ in the presence of Na^+, K^+, choline, Li^+ and mannitol, respectively). In a related investigation, we tested the effect of pretreating hRPE cells (for 30 min at 37°C) with ouabain (a Na^+–K^+-ATPase inhibitor; 1 mM), on the initial rate of RF (8 nM) uptake. No effect on RF uptake was observed by such a treatment (uptake of 344.3 ± 3.8 and 343.7 ± 5.0 fmol (mg protein)$^{-1}$ (4 min)$^{-1}$ in the presence and absence of ouabain, respectively).

Evidence for involvement of a carrier-mediated mechanism in RF uptake by hRPE cells

Figure 3 depicts the results of the initial rate of uptake of RF as a function of increasing the substrate concentration (8 nM to 100 μM) in the incubation medium. As can be seen, uptake was saturable as a function of concentration within the range of 8 nM and 1 μM with an apparent K_m of 80 ± 14 nM and a V_{max} of 1.80 ± 0.10 pmol (mg protein)$^{-1}$ (4 min)$^{-1}$. These findings suggest the involvement of a carrier-mediated mechanism in RF uptake by hRPE cells. At higher concentrations (> 5 μM) uptake was linear ($r = 0.99$, data not shown). To confirm the existence of a carrier-mediated system, we examined possible *trans*-stimulation in ^{3}H-RF transport by unlabelled RF. In this experiment, we first preloaded the cells with ^{3}H-RF (by incubation with 8 nM ^{3}H-RF for 10 min), then incubated the cells (for 10 min) in Krebs-Ringer buffer in the presence and absence of 20 μM unlabelled RF. The results showed the cell content of ^{3}H radioactivity to be significantly ($P < 0.01$) lower in hRPE cells incubated in the presence of unlabelled RF in the incubation medium compared to those incubated in buffer alone (cell content of ^{3}H radioactivity was 498.3 ± 6.2 and 370.1 ± 1.3 fmol

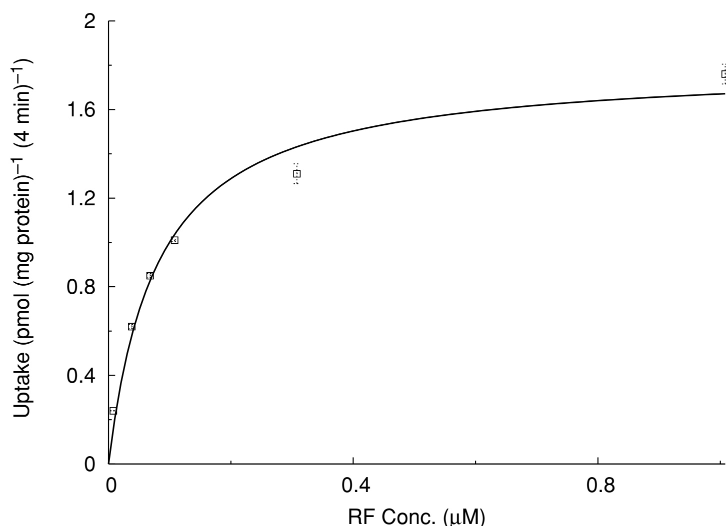

Figure 3. Uptake of RF by hRPE cells as a function of concentration
Confluent monolayers of hRPE were incubated for 4 min in Krebs-Ringer buffer pH 7.4, at 37°C in the presence of different concentrations of RF. Data are mean ± S.E.M. of 3–4 separate uptake determinations.

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Table 1. Effect of riboflavin (RF) structural analogues and related compounds on the uptake of ^{3}H-RF by hRPE cells

Compound	Conc. (μM)	^{3}H-RF uptake (fmol (mg protein)$^{-1}$ (4 min)$^{-1}$)	P value
A. Na$^+$-containing Krebs-Ringer buffer			
Control	—	285.8 ± 4.2	
Unlabelled RF	0.05	148.2 ± 3.2	< 0.01
	1	18.2 ± 0.6	< 0.01
Lumiflavine	0.1	153.5 ± 2.4	< 0.01
	1	68.6 ± 1.8	< 0.01
	5	13.2 ± 0.6	< 0.01
	10	8.1 ± 0.2	< 0.01
Lumichrome	1	204.4 ± 11.5	< 0.01
	5	90.1 ± 4.7	< 0.01
	10	61.8 ± 1.2	< 0.01
Lumazine	100	287.8 ± 3.5	n.s.
Thiamin	25	286.9 ± 1.6	n.s.
B. K$^+$-containing Krebs-Ringer buffer			
Control	—	285.0 ± 11.3	
Lumiflavine	1	67.7 ± 1.3	< 0.01
	10	9.4 ± 0.7	< 0.01
Lumichrome	1	194.3 ± 8.8	< 0.01
	10	71.7 ± 3.3	< 0.01

Monolayers of hRPE cells were incubated at 37°C in either Na$^+$- or K$^+$-containing Krebs-Ringer buffer. ^{3}H-RF (8 nM) and the compound under investigation were added at onset of 4 min incubation. Data are mean ± S.E.M. of 3–4 separate uptake determinations. n.s., not significant. P value was calculated using Student's t test with comparison being made relative to simultaneously performed controls.

(mg protein)$^{-1}$, respectively). A similar *trans*-stimulation study was performed using lumiflavine (20 μM) in the incubation buffer to induce ^{3}H-RF efflux from preloaded cells. The results again showed that the cell content of ^{3}H radioactivity was significantly ($P < 0.01$) lower in cells incubated in the presence of lumiflavine in the incubation medium compared to those incubated in buffer alone (cell content of ^{3}H radioactivity was 513.0 ± 0.2 and 381.5 ± 6.3 fmol (mg protein)$^{-1}$, respectively).

In other studies, we determined if *cis*-inhibition occurs in the initial rate of ^{3}H-RF (8 nM) uptake by hRPE cells upon the addition of unlabelled RF and its structural analogues and related compounds to the Na$^+$-containing Krebs-Ringer incubation buffer. The results (Table 1) showed that the RF structural analogues lumiflavine and lumichrome caused a significant and concentration-dependent inhibition of the initial rate of ^{3}H-RF uptake (Table 1). On the other hand, no effect was observed when the RF-related compound lumazine and the unrelated thiamin were added to the incubation medium (Table 1). We also examined the effect of the RF structural analogues lumiflavine and lumichrome on ^{3}H-RF uptake by monolayers of hRPE cells incubated in a K$^+$-containing Krebs-Ringer incubation buffer (i.e. no Na$^+$) and found that the two compounds caused a similar degree of inhibition in the initial rate of ^{3}H-RF uptake (8 nM) to that seen in the cells incubated in Na$^+$-containing buffer. Taken together, these findings demonstrated the existence of a carrier-mediated mechanism for RF uptake by the hRPE cells.

Effect of inhibitors of membrane transport and sulfhydryl groups on RF uptake by hRPE cells

The effects of the membrane transport inhibitors DIDS, SITS and amiloride (all at 1 mM) on the initial rate of RF (8 nM) uptake were examined. All inhibitors tested caused a significant ($P < 0.01$ for all) inhibition in RF uptake (297.7 ± 2.7, 146.5 ± 4.4, 220.9 ± 4.1 and 224.8 ± 5.7 fmol (mg protein)$^{-1}$ (4 min)$^{-1}$ for control and in the presence of DIDS, SITS and amiloride, respectively). In a separate study, we examined the effect of pretreating (for 30 min) the cells with the sulfhydryl group inhibitor *p*-chloromercuriphenylsulphonate (*p*-CMPS, 0.05 mM) on the initial rate of RF (8 nM) uptake. The results showed significant inhibition in the initial rate of RF uptake by such a treatment with the inhibitory effect being significantly ($P < 0.01$) reversed following treatment of the cells with the reducing agent dithiothreitol (10 mM) for 30 min (318.8 ± 4.6, 41.4 ± 1.5 and 142.0 ± 1.2 fmol (mg protein)$^{-1}$ (4 min)$^{-1}$ for control cells, those pretreated with *p*-CMPS, and those pretreated with *p*-CMPS first and then with dithiothreitol, respectively).

Regulation of the RF uptake process of hRPE cells by extracellular and intracellular factors

In these studies, we examined the effect of maintaining the hRPE cells in a RF-deficient growth medium on uptake of ^{3}H-RF. We also investigated the possible role of specific intracellular regulatory pathways in the regulation of the RF uptake process by these cells. Maintaining the cells in a RF-deficient growth medium for 24 h was found to cause a significant ($P < 0.01$) induction in the initial rate of ^{3}H-RF (8 nM) uptake (495.1 ± 11.7 and 263.8 ± 2.8 fmol (mg protein)$^{-1}$ (4 min)$^{-1}$ for cells grown in deficient and control growth media, respectively). This effect was specific as uptake of the unrelated [^{3}H]thiamin (8 nM) was found to be similar in the two cell groups (249.4 ± 5.8 and 244.9 ± 8.4 fmol (mg protein)$^{-1}$ (4 min)$^{-1}$ for cells grown in deficient and control growth media, respectively). In contrast to the effect of RF deficiency, growing the hRPE cells for 24 h in RF-over-supplemented growth medium (20- and 60-fold above that of control) led to a decrease in the initial rate of ^{3}H-RF (8 nM) uptake (240.7 ± 2.4, 206.9 ± 2.3 and 155.7 ± 5.2 fmol (mg protein)$^{-1}$ (4 min)$^{-1}$ for cells grown in control and in the presence of 20- and 60-fold excess RF, respectively).

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To determine whether or not the effect of the RF-deficient conditions on ^{3}H-RF uptake is mediated via an effect on the V_{max} and/or the apparent K_m of the RF uptake process, we examined the uptake as a function of RF concentration in cells grown under the two different conditions. The results (Fig. 4) showed that growing the cells under RF-deficient conditions led to a significant ($P < 0.01$) induction in V_{max} (2.43 ± 0.05 and 1.81 ± 0.05 pmol (mg protein)$^{-1}$ (4 min)$^{-1}$ for cells grown in RF-deficient and control media, respectively) and a significant ($P < 0.01$) decrease in the apparent K_m (45 ± 3 and 73 ± 7 nM for cells grown in RF-deficient and control media, respectively) of the RF uptake process.

The possible role of intracellular regulatory pathways in the regulation of RF uptake by hRPE was examined using modulators of specific signalling pathways. We focused on examing the role of the Ca^{2+}–calmodulin-, protein kinase A (PKA)-, protein kinase C (PKC)-, and nitric oxide (NO)-mediated pathways based on previous studies from different laboratories showing that these pathways play a role in regulating nutrient uptake by different epithelia (Rood *et al.* 1988; Cohen *et al.* 1990; Brandsch *et al.* 1993; Piper *et al.* 1993; Donowitz *et al.* 1994; Gill *et al.* 2002). Our findings showed that pretreating the hRPE cells (for 1 h) with calmidazolium (25 μM), an inhibitor of the Ca^{2+}–calmodulin-mediated pathway, resulted in a significant ($P < 0.01$) concentration-dependent inhibition in RF uptake (291.3 ± 8.6, 257.6 ± 12.1, 214.0 ± 3.2, 147.6 ± 7.1 and 26.7 ± 1.0 fmol (mg protein)$^{-1}$ (4 min)$^{-1}$ for control and the presence of 10, 20, 25 and 50 μM calmidazolium, respectively). Similarly, pretreating the cells with trifluoperazine (TFP; 50 μM), another inhibitor of the Ca^{2+}–calmodulin-mediated pathway, caused a significant ($P < 0.01$ for both) inhibition in RF (8 nM) uptake (319.7 ± 5.6 and 217.3 ± 5.5 fmol (mg protein)$^{-1}$ (4 min)$^{-1}$ for control and the presence of 50 μM TFP, respectively). We also determined if the effect of calmidazolium (25 μM) on RF uptake was mediated via an effect on the V_{max} and/or the apparent K_m of the RF uptake process. Our findings (Fig. 5) showed that the effect was mediated via a significant ($P < 0.01$) decrease in the V_{max} (1.79 ± 0.08 and 1.14 ± 0.04 pmol (mg protein)$^{-1}$ (4 min)$^{-1}$ for control and in calmidazolium-pretreated cells, respectively), and a significant ($P < 0.01$) increase in the apparent K_m (72 ± 12 and 130 ± 16 nM, for control and in calmidazolium-pretreated cells, respectively) of the RF uptake process.

The potential role for the PKA-mediated pathway in the regulation of RF uptake by hRPE was also examined by testing the effect of pretreating (for 1 h) the cells with dibutyryl cAMP (1 mM) or 8-bromo-cAMP (1 mM). Neither compound was found to have a significant effect on RF uptake (270.1 ± 1.5, 269.2 ± 8.6 and 272.0 ± 7.2 fmol (mg protein)$^{-1}$ (4 min)$^{-1}$ for control, dibutyryl cAMP and 8-bromo-cAMP, respectively). Similarly, no role for the PKC-mediated pathway was apparent as pretreatment with modulators of this pathway failed to affect the initial rate of RF (8 nM) uptake by hRPE cells (270.2 ± 5.8, 266.8 ± 6.0 and 275.1 ± 4.7 fmol (mg protein)$^{-1}$ (4 min)$^{-1}$ for control and in the presence of 1 μM phorbol 12-myristate 13-acetate (PMA), and 10 μM chelerythrine, respectively). A role for the PTK-mediated pathway was also investigated by testing the effect of pretreatment (for 1 h) with genistein (50 μM) and tyrophostin A-1 (50 μM). The results showed no significant effect on RF uptake by either compound (279.6 ± 0.9, 275.5 ± 3.7 and 284.9 ± 3.5 fmol (mg protein)$^{-1}$ (4 min)$^{-1}$ for control and following pretreatment with genistein and tyrophostin A-1, respectively). Finally, we tested the potential role of the NO-mediated pathway in the regulation of RF uptake by hRPE cells. This was performed by examining the effect of pretreating (1 h) the cells with modulators of

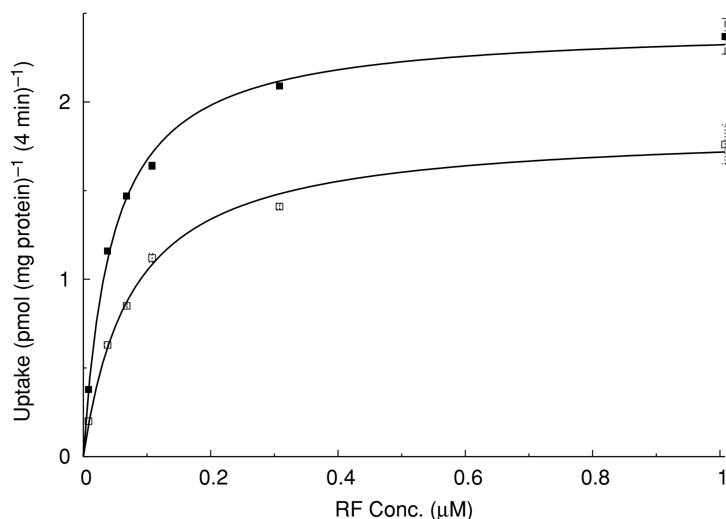

Figure 4. Effect of RF deficiency on RF uptake by hRPE cells

Confluent monolayers of hRPE were maintained for 24 h in a RF-deficient (■) or RF-sufficient, i.e. control (□), growth medium (see Methods). Initial rate of uptake of different concentrations of RF was then examined in the two cell types incubated in Krebs-Ringer buffer pH 7.4. Data are mean ± S.E.M. of 3–4 separate uptake determinations.

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this pathway on the initial rate of RF (8 nM) uptake. The results showed that RF uptake was not affected by S-Nitrose-N-acetylpenicillamine (SNAP; 1 mM), Sodium nitroprusside (SNP; 0.5 mM) or 8-bromo cGMP (0.5 mM) (305.1 ± 2.7, 303.2 ± 2.2, 306.3 ± 5.1 and 301.7 ± 1.6 fmol (mg protein)$^{-1}$ (4 min)$^{-1}$ for control and following pretreatment with SNAP, SNP and cGMP, respectively).

Discussion

The aim of the present study was to determine the mechanism and regulation of RF uptake by the hRPE using the cultured ARPE-19 cell line as a human retinal pigment epithelial model system. RF is important for the normal function of all mammalian cells especially those with high metabolic rates such as those of the retina. The retina obtains its supply of RF mainly from the choroidal circulation via transport across the retinal pigment epithelial cells. Thus, the normal function of these cells is important for the health of the retina. Using monolayers of the hRPE in culture, we found RF uptake to occur without any metabolic alterations in the uptake and to be both temperature- and energy-dependent in nature. RF uptake was also found be Na^+ independent because replacing Na^+ in the incubation medium with other monovalent cations or with the inert mannitol did not affect the vitamin uptake by the hRPE cells. The inability of the Na^+–K^+-ATPase inhibitor ouabain to inhibit RF uptake further confirms the Na^+-independent nature of the uptake process. The uptake process of RF, however, was found to be pH dependent and decreased as a function of decreasing the incubation buffer pH from 7.4 to 5.0. The mechanism through which extracellular buffer pH influences RF uptake is not clear but similar findings have been previously described in the human-derived liver HepG2 cells (Said *et al.* 1998).

Evidence for the involvement of a carrier-mediated mechanism for RF uptake by the hRPE cells was also obtained. Saturation in the initial rate of RF uptake as a function of concentration was found with an apparent K_m of the saturable process of 80 ± 14 nM. In addition, unlabelled RF and its structural analogue lumiflavine both caused significant *trans*-stimulation in ^{3}H-RF transport. Furthermore, unlabelled RF, lumiflavine and lumichrome all caused a significant and concentration-dependent *cis*-inhibition in the initial rate of ^{3}H-RF uptake by these cells. The inability of the related compound lumazine and the unrelated vitamin thiamin to inhibit the initial rate of uptake of RF demonstrates the specificity of the RF uptake process of the hRPE cells. As had been discussed in previous reports using monolayers of hRPE cells in transport investigations (Huang *et al.* 1997; Aukunuru *et al.* 2001), the polarity of these cells is not clear, and thus, we are unable to conclude with certainty the cell membrane domain(s) at which the identified RF carrier system is functional. Further studies using hRPE cells grown on permeable support are needed to address this issue.

Previous studies have suggested that RF behaves as an anion with regards to its transport across cell membranes (Spector, 1982; Lowy & Spring, 1990). Thus, we examined the effect of the anion transport inhibitors DIDS and SITS on RF uptake by the hRPE cells. Our data indicated a significant inhibition in substrate uptake in the presence of these transport inhibitors, providing support for the above suggestion. The ability of the pyrazine diuretic amiloride (an inhibitor of Na^+–H^+ exchange) to inhibit RF uptake by hRPE was similar to what has been observed previously for the substrate uptake by other epithelia (Said & Ma, 1994; Said *et al.* 2000). This raises the possibility that a drug–vitamin interaction may occur at the level of the cell membrane, and suggests a need for *in vivo* investigations to further address this issue. The uptake process of RF was

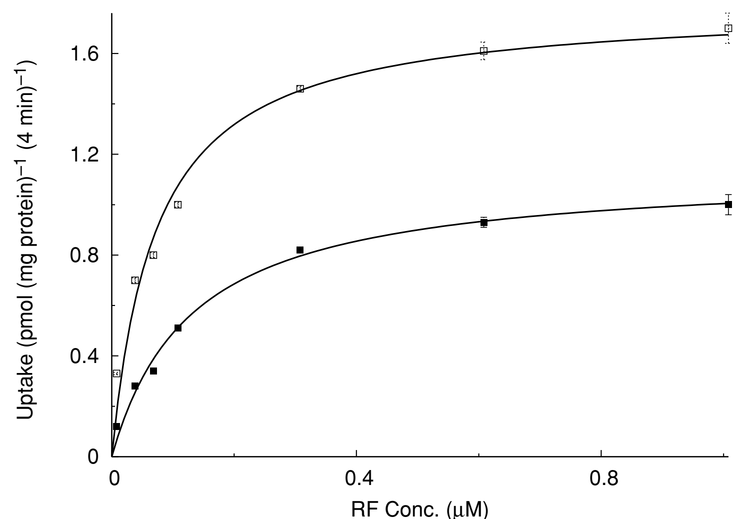

Figure 5. Effect of pretreatment of hRPE cells with calmidazolium on uptake of RF as a function of concentration
Confluent monolayers of hRPE were pretreated for 1 h with 25 μM calmidazolium (■) or with buffer (□). Initial rate of uptake of different concentrations of RF was then examined in the two cell types incubated in Krebs-Ringer buffer pH 7.4. Data are mean ± S.E.M. of 3–7 separate uptake determinations.

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also found to be sensitive to the effect of the -SH group inhibitor *p*-CMPS, suggesting a possible involvement of such groups in the vitamin uptake process. The ability of the reducing agent dithiothreitol to significantly reverse the inhibitory effect of *p*-CMPS on RF uptake by hRPE confirms the notion that this inhibitor is interacting with -SH groups. Since *p*-CMPS is membrane impermeant, it is reasonable to suggest that the -SH groups with which this compound is interacting are located at the exofacial domain of the hRPE cells.

After identifying the uptake mechanism involved in RF uptake by the hRPE cells, we examined the possible regulation of the RF uptake process by extracellular and intracellular factors. Our results showed that maintaining hRPE cells in a RF-deficient growth medium leads to a specific and significant up-regulation in the initial rate of uptake of ^{3}H-RF. This increase was mediated by an induction in the V_{max} and a decrease in the apparent K_m of the RF uptake process. These findings suggested that RF deficiency was associated with an increase in the number (and/or activity) and the affinity of the RF transporters, respectively. Further studies using molecular probes (which are not currently available) should assist in delineating the molecular mechanism(s) involved in such adaptive regulation. We also investigated the possible regulation of the RF uptake process of the hRPE cells by intracellular regulatory pathways. We focused on the role of the Ca^{2+}–calmodulin-, PKA-, PKC-, PTK- and NO-mediated pathways, as these pathways have been shown to play an important role in regulating the transport of other substrates in different epithelial cell types (Rood *et al.* 1988; Cohen *et al.* 1990; Brandsch *et al.* 1993; Piper *et al.* 1993; Donowitz *et al.* 1994; Gill *et al.* 2002). We used specific modulators of the various signalling pathways in our investigations. Our results showed that while no roles for PKA-, PKC-, PTK- and NO-mediated pathways in RF uptake were evident, a role for the Ca^{2+}–calmodulin-mediated pathway was apparent. Modulators of the latter pathway were found to cause a significant inhibition in RF uptake, and the effect (at least for calmidazolium) appeared to be mediated via a significant decrease in V_{max} and a significant increase in the apparent K_m of the RF uptake process. The latter findings, which are similar to those seen for the vitamin uptake in intestinal, renal and hepatic epithelial cells (Kumar *et al.* 1998; Said *et al.* 1998, 2000), suggest that the effect of calmidazolium is mediated via a decrease in the activity (and/or number) and the affinity of the RF uptake process. The cellular mechanism(s) through which the Ca^{2+}–calmodulin-mediated pathway exerts its effect on RF uptake is(are) not clear but different mechanisms for the action of this pathway have been described that include the activation of specific protein kinase(s) and a potential direct effect on the uptake system involved.

In summary, the results of the present study demonstrate for the first time the involvement of a specialized, high-affinity carrier-mediated mechanism for RF uptake by hRPE cells. In addition, the study shows that this system is up-regulated in RF deficiency and modulated by an intracellular Ca^{2+}–calmodulin-mediated pathway.

References

Aukunuru JV, Sunkara G, Bandi N, Threson WB & Kompella UB (2001). Expression of multi-drug resistance-associated protein (MRP) in human retinal pigment epithelial cells and its interaction with BAPSG, a novel aldose reductase inhibitor. *Pharm Res* **18**, 565–572.

Batey DW, Daneshgar KK & Eckhert CD (1992). Flavin levels in the rat retina. *Exp Eye Res* **54**, 605–609.

Blot WJ, Li JY, Taylor PR, Guo W, Dawsey S, Wang GQ, Yang CS, Zheng SF, Gail M & Li GY (1993). Nutrition intervention trials in Linxian, China. Supplementation with specific vitamin/mineral combinations; cancer incidence and disease-specific mortality in the general population. *J Natl Cancer Inst* **85**, 1483–1492.

Brandsch M, Miyamoto Y, Ganapathy V & Leibach FH (1993). Regulation of taurine transport in human colon carcinoma cell line (HT-29 and Caco-2) by protein kinase C. *Am J Physiol* **264**, G939–G946.

Busik JV, Olson LK, Grant MB & Henry DN (2002). Glucose-induced activation of glucose uptake in cells from the inner and outer blood–retinal barrier. *Invest Ophthalmol Vis Sci* **43**, 2356–2363.

Chancy CD, Kekuda R, Huang W, Prasad PD, Kuhel JM, Sirotnak FM, Roon P, Ganapathy V & Smith SB (2000). Expression and differential polarization of the reduced-folate transporter-1 and the folate receptor α in mammalian retinal pigment epithelium. *J Biol Chem* **275**, 20676–20684.

Cohen ME, Reinlib L, Watson AJM, Gorelick F, Sikora KR, Tse M, Rood RP, Czernik AJ, Sharp GW & Donowitz M (1990). Rabbit ileal villus cell brush border Na^+/H^+ exchanger is regulated by Ca^{2+}/calmodulin-dependent protein kinase II, a brush border membrane protein. *Proc Natl Acad Sci U S A* **87**, 8990–8994.

Cooperman JM & Lopez R (1984). Riboflavin. In *Handbook of Vitamins: Nutritional, Biochemical and Clinical Aspects*, ed. Machlin LJ, pp. 299–327. Marcel Dekker, New York.

Cumming RG, Michell P & Smith W (2000). Diet and cataract: the blue mountains eye study. *Ophthalmology* **107**, 450–456.

Donowitz M, Montgomery JLM, Walker MS & Cohen ME (1994). Brush border tyrosine phosphorylation stimulates ileal neutral NaCl absorption and brush-border Na^+/H^+ exchange. *Am J Physiol* **266**, G647–G656.

Gill RK, Saksena S, Syed IA, Tyagi S, Alrefui WA, Malakooti J, Ramasmamy K & Dudeja PK (2002). Regulation of NHE-3 by nitric oxide in Caco-2 cells. *Am J Physiol Gastrointest Liver Physiol* **283**, G747–G756.

Goldsmith GA (1975). Riboflavin deficiency. In *Riboflavin*, ed. Rivlin RS, pp. 221–244. Plenum, New York.

Huang W, Prasad PD, Kekuda R, Leibach FH & Ganapathy V (1997). Characterization of N5-methyltetrahydrofolate uptake in cultured human retinal pigment epithelial cells. *Invest Ophthalmol Vis Sci* **38**, 1578–1587.

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Kumar CK, Yanagama N, Ortiz A & Said HM (1998). Mechanism and regulation of riboflavin uptake by human renal proximal tubule epithelial cell line Hk-2. *Am J Physiol* **274**, F104–F110.

Lowry OH, Rosenbrough NJ, Farr AJ & Randall RJ (1951). Protein measurements with the Folin phenol reagent. *J Biol Chem* **193**, 265–275.

Lowy RJ & Spring KR (1990). Identification of riboflavin transport by MDCK cells using quantitative fluorescence video microscopy. *J Membr Biol* **117**, 91–99.

Merrill AJ, Lambeth JD, Edmondson D & McCormick DB (1981). Formation and mode of action of flavoproteins. *Annu Rev Nutr* **1**, 281–317.

Miyamota Y & Sancar A (1998). Vitamin B_2 based blue photoreceptors in the retinohypothalamic tract as the photoactive pigments for setting the circadian clock in mammals. *Proc Natl Acad Sci U S A* **95**, 6097–6102.

Piper RC, James DE, Slot JW, Puri C & Lawrence JC Jr (1993). GLUT4 phosphorylation and inhibition of glucose transport by dibutyryl cAMP. *J Biol Chem* **268**, 16557–16563.

Pow DV (2001). Amino acids and their transporters in the retina. *Neurochem Int* **38**, 463–484.

Rao VV, Dahlheimer JL, Bardgett ME, Snyder AZ, Finch RA & Piwnica WD (1999). Choroid plexus epithelial expression of MDRI P-glycoprotein and multidrug resistance-associated protein contribute to the blood–cerebrospinal fluid permeability barrier. *Proc Natl Acad Sci U S A* **96**, 3900–3905.

Rood RP, Emmer E, Wesoleck J, McCullen J, Husain Z, Cohen ME, Braithwaite RS, Murer H, Sharp GW & Donowitz M (1988). Regulation of the rabbit ileal brush-border Na^+/H^+ exchanger by an ATP-requiring Ca^{2+}/calmodulin-mediated process. *J Clin Invest* **82**, 1091–1097.

Said HM & Ma TY (1994). Mechanism of riboflavin uptake by Caco-2 human intestinal epithelial cells. *Am J Physiol* **266**, G15–G21.

Said HM, Ortiz A, Ma TY & McCloud E (1998). Riboflavin uptake by the human-derived liver cells Hep G2. Mechanisms and regulation. *J Cell Physiol* **176**, 588–594.

Said HM, Ortiz A, Moyer MP & Yanagawa N (2000). Riboflavin uptake by human-derived colonic epithelial NCM460 cells. *Am J Physiol Cell Physiol* **278**, C270–C276.

Spector R (1982). Riboflavin transport by rabbit kidney slices: characterization and relation to cyclic organic acid transport. *J Pharmacol Exp Ther* **221**, 394–398.

Takami Y, Gong H & Amemiya T (2004). Riboflavin deficiency induces ocular surface damage. *Ophthalmic Res* **36**, 156–165.

Wilkinson GN (1961). Statistical estimation in enzyme kinetics. *Biochem J* **80**, 324–332.

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