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Riboflavin uptake by rat liver basolateral membrane vesicles

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Abstract

The present study examined riboflavin (RF) uptake by purified rat liver basolateral membrane vesicle (BLMV). Uptake of RF was found to be Na^+-and pH-independent in nature. Studies on RF uptake by BLMV as a function of incubation medium osmolarity have indicated that the uptake is the result of transport (66.5%) into the intravesicular space as well as binding (33.5%) to membrane surfaces. The process of RF uptake by BLMV was saturable as a function of substrate concentration with an apparent K_m of 3.55 ± 0.70 μM and V_{max} of 39.89 ± 3.24 pmol/mg protein/5 s, respectively. *cis*-Addition of unlabeled RF and its structural analogs lumaflavin and lumichrome inhibited the uptake of [^{3}H]RF while *trans*-addition of unlabeled RF stimulated the efflux of [^{3}H]RF from preloaded vesicles. No effect on RF uptake was found by the membrane transport inhibitors probenecid, 4,4-diisothiocyanotostilbene-2,2-disulfonic acid (DIDS) and 4-acetamido-4-isothiocyanatostilbene-2,2′-disulfonic acid (SITS). Induction of a transient positive intravesicular space led to a slight stimulation of RF uptake, while induction of a negative intravesicular space led to a slight inhibition in RF uptake. These results demonstrate the existence of a membrane-associated carrier system for RF uptake by liver BLMV. This system appears to be Na^+- and pH-independent and is influenced to a certain degree by changes in transmembrane electrical potential.

Keywords: Riboflavin transport; Membrane vesicle; (Rat liver)

1. Introduction

Riboflavin (RF) is a water-soluble vitamin, that is essential for normal cellular functions, growth and development [1–3]. In its coenzyme forms riboflavin 5′-phosphate (FMN) and flavin adenine dinucleotide (FAD), RF serves in key metabolic reactions including carbohydrate, amino acid and lipid metabolism, as well as in the conversion of pyridoxine into its coenzyme forms. Furthermore, a role for RF in the protection of tissues against oxidative injury has been recently advanced [4].

The liver plays an important role in normal RF homeostasis and is a major site of RF metabolism and utilization. Hepatocytes obtain RF from the circulation via transport across the basolateral membrane (BLM). Limited studies are available describing the mechanism and regulation of RF uptake across the hepatocyte BLM. Aw et al. have utilized isolated rat hepatocytes in suspension and suggested the involvement of metabolic trapping (i.e., phosphorylation) in the uptake process [5]. Although a useful technique, isolated hepatocytes in suspension cannot differentiate between uptake across the BLM domain of the polarized hepatocyte and that which takes place across its canalicular membrane domain. Also, use of this preparation cannot eliminate the influence of substrate intracellular metabolism, i.e., metabolic trapping, of a substrate after uptake into the cell. In the case of RF, the liver contains significant flavokinase activity (which converts RF to FMN) and FAD synthetase activity (which converts FMN to FAD) [6,7]. Both of the above described complications could, however, be significantly eliminated with the use of purified liver BLM vesicles (BLMV). In this study we therefore utilized this technique to study RF transport into liver cells, with special emphasis on examining the existence of a specialized BLM-associated uptake system.

2. Materials and methods

[G-^{3}H]Riboflavin (spec. act. 40 Ci/mmol; radiochemical purity = 98%) was obtained from American Radiola-

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beled Chemicals, St. Louis, MO. Unlabeled riboflavin, lumiflavin and lumichrome were purchased from Sigma, St. Louis. All other chemicals and reagents were obtained from commercial sources and were of analytical quality. Cellulose nitrate filters (pore size of 0.45 μm) were purchased from Millipore, Bedford, MA.

BLMV were isolated from the liver of Sprague-Dawley rats (Sasco, Omaha, NE) using a modification of Blitzer and Donovon's [8] self-generating Percoll gradient method described by us in detail previously [9]. Isolated liver vesicles were suspended in an uptake (intravesicular) buffer of 280 mM mannitol and 20 mM Hepes/Tris, pH 7.4 to an average protein concentration of 2.50 ± 0.19 mg/ml. Uptake experiments were performed on freshly isolated BLMV utilizing a rapid-filtration technique [10] as described by us before [11], and were initiated by mixing 20 μl of BLMV suspension with 80 μl of incubation buffer (in mM: NaCl or KCl 100, mannitol 80, Hepes/Tris 20, pH 7.4) containing labeled and unlabeled RF. Incubation was terminated at the desired time by the addition of 1 ml of ice-cold stop solution (in mM: NaCl 100, mannitol 100, KH_2PO_4 10, pH 7.4) and the suspension was filtered then washed with 5 ml of ice-cold buffer. The filter was then dissolved in scintillation fluid and counted for radioactivity. In the transstimulation study, liver BLMV were first preloaded with [^{3}H]RF (5 μM) for 20 min at room temperature, pelleted, washed with ice-cold buffer then suspended in ice-cold transport buffer. Vesicles were then incubated at 37° C for 30 s with 80 μl of incubation buffer in the presence and absence of 100 μM unlabeled RF. At the end of incubation, the reaction was stopped, the vesicles were filtered and their radioactive content was determined.

The purity and suitability of rat liver BLMV for uptake studies have been demonstrated previously in our laboratory by morphological (electron microscopy), enzymatic and functional criteria [9]. Vesicle orientation was also established by freeze-fracture electron microscopy and by [^{3}H]ouabain binding assay [9]. Protein concentrations of membrane vesicles were measured by the method of Lowry et al. [12] using BSA as the protein standard. Each data point presented in this communication is the means ± S.E. of multiple separate uptake measurements from at least two different vesicle preparations from different rats and is expressed as pmol/mg protein/unit time. Data was analyzed using regression analysis and the Student's t-test. As seen before with this membrane preparation [13,14], some quantitative variations in the absolute amount of RF uptake was noticed in some BLMV preparations. For this reason, appropriate controls were run with each experiment to permit comparison. Kinetic parameters of the observed saturable component of RF uptake (i.e., the V_{max} and the apparent K_m) were calculated using a computerized model of the Michaelis-Menten equation as described by Wilkinson [15], following subtraction of the diffusion (and nonspecific) component from total uptake. Contribution of diffusion to total uptake was determined by examining RF uptake in the presence of a high concentration of lumiflavin (330 μM) which causes a similar degree of inhibition of the uptake as RF, see Results). The purity of the stock [^{3}H]RF and the metabolic form of the radioactivity taken up by liver BLMV following incubation with [^{3}H]RF were determined using silica-gel precoated TLC plates and a solvent system of ethanol and water (9:1, v/v) [16].

3. Results

3.1. General characteristics of RF uptake by liver BLMV

Fig. 1 depicts the uptake of 0.11 μM [^{3}H]RF as a function of time in liver BLMV incubated in Na^+ and K^+ containing buffers (outside = 100 mM, inside = 0 mM). Following an initial rapid phase, uptake of RF increased linearly for up to 20 s of incubation but leveled off thereafter reaching equilibrium after approx. 60 s of incubation. The initial rapid phase of uptake represented by the intersection of the linear uptake line with the y-axis most probably represents binding of RF to BLM (see below for more evidence). No difference was found in RF uptake by BLMV incubated in the presence of a Na^+ or a K^+ gradient. Similar results were found for the uptake of high concentration of RF (20 μM) with time (data not shown).

In another study, the effect of varying the incubation buffer pH on the uptake of RF (0.11 μM) by liver BLMV preloaded with transport buffer (pH 7.4) was examined. In these studies the buffering system of the incubation medium was either Hepes/Tris or Hepes/Mes. No effect of incubation buffer pH was found on RF uptake (uptake of 1.06 ± 0.06 ($n = 5$), 0.95 ± 0.07 ($n = 6$), 1.06 ± 0.08 (n

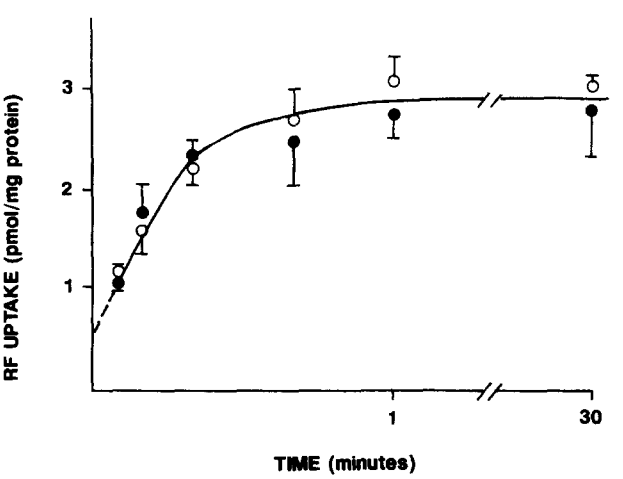

Fig. 1. Uptake of RF by rat liver BLMV as a function of time in the presence of a Na^+ or a K^+ gradient. BLMV were preloaded with a buffer of 280 mM mannitol and 20 mM Hepes/Tris pH 7.4. Incubation was done in an incubation buffer of 100 mM NaCl (closed circles) or KCl (open circles), 80 mM Mannitol and 20 mM Hepes/Tris pH 7.4. [^{3}H]RF (0.11 μM) was added to the incubation medium at the onset of incubation. Each data point represents means ± S.E. of three to eight determinations from three separate liver BLMV preparations.

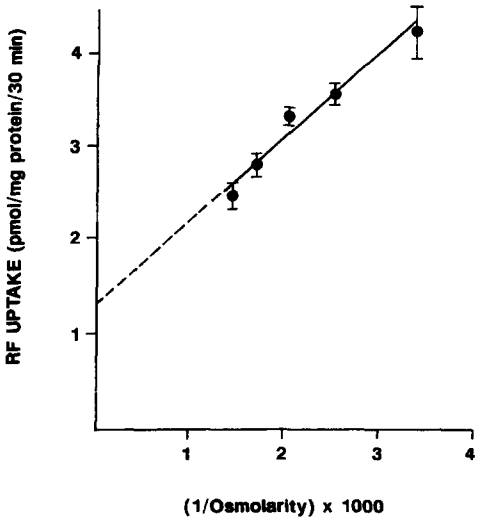

Fig. 2. Effect of incubation medium osmolarity on RF uptake by liver BLMV. Vesicles were preloaded with a buffer of 280 mM mannitol and 20 mM Hepes/Tris, pH 7.4. Incubation was performed for 30 min at 37° C in a buffer of 140 mM NaCl, 20 mM Hepes/Tris pH 7.4, and different amounts of mannitol. [^{3}H]RF (0.11 μM) was added to the incubation medium at the onset of incubation. Each data point represents means ± S.E. of three to six determinations from two separate liver BLMV preparations.

= 5), 1.03 ± 0.10 (n = 6), 1.12 ± 0.07 (n = 6), 1.00 ± 0.09 (n = 6) and 1.17 ± 0.09 (n = 6) pmol/mg protein/5 s at pH 5, 5.5, 6, 6.5, 7, 7.5, and 8, respectively).

Fig. 2 shows the uptake of RF (0.11 μM) by BLMV at equilibrium (30 min) as a function of changing the incubation medium osmolarity. An inverse and linear (r = 0.987) relationship was found between RF uptake and incubation medium osmolarity. Extrapolating the uptake line to infinite osmolarity intersected the y-axis at approx. 0.127. From this finding we calculated that the degree of binding of RF to BLM at equilibrium and under isoosmotic conditions to be 33.5% while the remaining 66.5% is due to transport into an osmotically active intravesicular space.

The metabolic form of the radioactivity taken up by liver BLMV following 30 min incubation with 0.33 μM [^{3}H]RF was also examined, using the TLC system described in Materials and methods. The result showed that the majority of the radioactivity (92.4%) taken up by the vesicles to be in the form of intact RF.

3.2. Involvement of a membrane-associated carrier system in RF uptake

To test for the involvement of a membrane-associated carrier system in the uptake of RF by liver BLMV, we examined the substrate uptake as a function of increasing the concentration in the incubation medium (0.11–10 μM). Uptake of RF by diffusion and non-specific uptake was determined, as described in Materials and methods, and was subtracted from total uptake at each RF concentration. Saturation was observed in RF uptake as a function of concentration (Fig. 3) suggesting the involvement of a specialized membrane associated system. The apparent K_m and V_{max} of the saturable uptake process were 3.55 ± 0.70 μM and 39.89 ± 3.24 pmol/mg protein/5 s, respectively.

To further examine the involvement of a membrane-associated carrier system for RF uptake by liver BLMV, we examined the effect of *cis*-addition of 50 μM unlabeled RF and the structural analogs lumiflavin and lumichrome on the uptake of 0.11 μM [^{3}H]RF. The results showed that all the three compounds caused significant ($P < 0.01$) inhibition in [^{3}H]RF uptake. The inhibition caused by unlabeled RF and lumiflavin (up to 82%) was higher than that caused by lumichrome (17%) (uptake of 1.62 ± 0.13 (n = 5), 0.32 ± 0.08 (n = 5), 0.30 ± 0.04 (n = 6) and 1.34 ± 0.08 (n = 5) pmol/mg protein/5 s for control and in the presence of unlabeled RF, lumiflavin, and lumichrome, respectively).

In another study, we tested for possible stimulation of [^{3}H]RF efflux from pre-loaded BLMV by *trans*-addition of unlabeled RF as described in Materials and methods. The results showed significantly ($P < 0.01$) lower [^{3}H]RF content in BLMV incubated in the presence of unlabeled RF in the incubation medium compared to those incubated in its absence (vesicles content of [^{3}H]RF of 6.8 ± 1.35 (n = 5) and 19.16 ± 2.1 (n = 6) pmol/mg protein after 30 s incubation, respectively).

3.3. Effect of membrane transport inhibitors and transmembrane electrical potential

The effect of the membrane transport inhibitors, probenecid, DIDS and SITS (all at 1 mM), on the uptake of 0.11 μM RF by liver BLMV was examined. None of

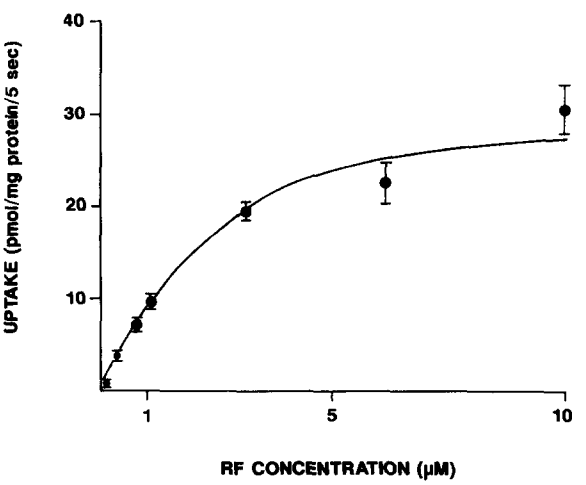

Fig. 3. Uptake of RF by liver BLMV as a function of concentration. BLMV were preloaded with a buffer of 280 mM mannitol and 20 mM Hepes/Tris, pH 7.4. Incubation was performed in a buffer of 140 mM NaCl and 20 mM Hepes/Tris, pH 7.4. Different concentrations of RF were added to the incubation medium at the onset of incubation. Each data point represents means ± S.E. of four to seven determinations from three separate liver preparations.

the inhibitors tested was found to affect RF uptake (uptake of 1.14 ± 0.04 ($n = 5$) 1.10 ± 0.04 ($n = 5$), 1.37 ± 0.12 ($n = 5$) and 1.07 ± 0.11 ($n = 5$) pmol/mg protein/5 s for control and in the presence of probenecid, DIDS and SITS, respectively).

In other studies, we examined the effect of altering transmembrane electrical potential on the uptake process of RF by liver BLMV. Two well established methods were used: an anion substitution method and a valinomycin-induced K^+-diffusion potential [9,17,18]. In the first method, uptake of 0.11 μM RF was examined in the presence of anions of different lipid (i.e., membrane) permeability ($SCN^- > NO_3^- >$ gluconate$^-$). In such a study, incubation with a lipid permeant anion would create a relatively larger transient negative intravesicular electrical potential than a relatively impermeant anion. The results showed a trend of an increase in RF uptake with the decrease in anion membrane permeability (uptake of 1.69 ± 0.17 ($n = 5$), 1.85 ± 0.06 ($n = 5$) and 2.61 ± 0.11 ($n = 7$) pmol/mg protein/5 s for SCN^-, NO_3^- and gluconate$^-$, respectively). This suggests that the RF uptake by liver BLMV is sensitive to alteration in transmembrane electrical potential. This suggestion was further confirmed by the finding that induction of a transient negative intravesicular space, with the use of an outwardly directed K^+ gradient ($K_i^+ = 50$ mM, $K_o^+ = 0$ mM) and valinomycin (10 μg/mg protein), caused a slight (though not significant) decrease in RF uptake compared to control. The 'voltage clamp' condition ($K_i^+ = K_o^+ = 50$ mM plus valinomycin) resulted in a slight (though not significant) increase in RF uptake following induction of a transient positive intravesicular space with the use of an inwardly directed K^+ gradient ($K_i^+ = 0$ mM, $K_o^+ = 50$ mM) and valinomycin (uptake of 1.72 ± 0.38 ($n = 5$), 2.0 ± 0.25 ($n = 6$) and 2.26 ± 0.12 ($n = 5$) pmol/mg protein/5 s, respectively).

4. Discussion

The present study examined RF uptake by rat liver BLMV isolated by a well established technique. We used this liver preparation in order to eliminate the interference of RF metabolism by flavokinase and FAD synthetase (the liver contains significant activities of these enzymes [6,7]) with the uptake process. Furthermore, with this liver preparation the interference of uptake across the canalicular membrane domain, a problem associated with the use of isolated hepatocytes in suspension, was eliminated. Uptake of RF was occurred without metabolic alteration of the RF molecule confirming the suitability of this preparation. RF uptake by liver BLMV was found to be Na^+-independent in nature, as indicated by the similar uptake in BLMV incubated in the presence of imposed Na^+ and a K^+ gradients. This is in agreement with a previous observation made in isolated hepatocytes [5], and with recent findings for RF transport across plasma membranes of other cell types including those of mammalian small intestine and *Xenopus* laevis oocytes [13,16,19–21]. This may suggest a universal nature of RF uptake process across biological membranes. The uptake of RF was also found to be pH-independent as indicated by the lack of an effect of incubation buffer pH on the substrate uptake. The study on the effect of incubation medium osmolarity on RF uptake revealed that the uptake process is a composed of transport into an osmotically active intravesicular space (66.5%), and binding to membrane surfaces (33.5%). In this study, our focus was to characterize the overall uptake process of RF by BLMV with a special emphasis on the existence of a BLM-associated uptake system.

The uptake process of RF by liver BLMV involved a specialized membrane-associated carrier system. Evidence for this includes the saturation in RF uptake as a function of increasing the substrate concentration in the incubation medium, *cis*-inhibition in [^{3}H]RF uptake by unlabeled RF and its structural analogs lumiflavin and lumichrome, and *trans*-stimulation of [^{3}H]RF efflux from pre-loaded BLMV by unlabeled RF in the incubation medium. The ability of unlabeled RF and lumiflavin, when added to the incubation medium, to cause up to 82% inhibition in [^{3}H]RF uptake indicates that both components of the uptake process, i.e., transport into the intravesicular spaces and binding to BLM, are affected by such treatments and are specific.

Previous studies have reported that probenecid may interfere with RF transport in the kidney, i.e., RF behaves like an anion in its transport characteristic [21]. For this reason, we tested the effect of probenecid on the uptake of [^{3}H]RF by liver BLMV. No effect, however, was seen, suggesting that RF does not utilize a probenecid-sensitive, general anion uptake system. This was further supported by the findings that the anion transport inhibitors DIDS and SITS also had no effect on RF uptake by liver BLMV.

The role of transmembrane electrical potential in RF uptake by liver BLMV was also investigated by two independent methods. A trend was found in that induction of a transient intravesicular positive intravesicular electrical potential causes slight stimulation in the uptake of RF compared to control values, while induction of a transient negative intravesicular electrical potential causes a slight inhibition in RF uptake by liver BLMV. Since RF exists in the neutral (uncharged) form at physiological pH values (pK_a of RF = 10.2; $pK_b = 1.7$) and since the uptake process for RF appears to be Na^+ and pH-independent, these findings suggest that transmembrane electrical potential may influence, to a certain degree, the activity of the putative RF uptake carrier itself.

In summary, the findings of the present study describe for the first time the mechanism of RF uptake across the liver BLM using BLMV and demonstrate the involvement of a membrane-associated carrier system. This system appears to be Na^+- and pH-independent and is influenced, to a certain degree, by alterations in transmembrane electrical potential.

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