Mechanism and regulation of folate uptake by human pancreatic epithelial MIA PaCa-2 cells

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Nabokina, Svetlana M., Thomas Y. Ma, and Hamid M. Said. Mechanism and regulation of folate uptake by human pancreatic epithelial MIA PaCa-2 cells. Am J Physiol Cell Physiol 287: C142–C148, 2004.—After the liver, the pancreas contains the second highest level of folate among human tissues, and folate deficiency adversely affects its physiological function. Despite that, nothing is currently known about the cellular mechanisms involved in folate uptake by cells of this important exocrine organ or about folate uptake regulation. We have begun to address these issues, and in this report we present the results of our findings on the mechanism of folate uptake by the human-derived pancreatic MIA PaCa-2 cells. Our results show folic acid uptake to be (1) temperature and energy dependent; (2) pH dependent, with a markedly higher uptake at acidic pH compared with neutral or alkaline pH; (3) Na+ independent; (4) saturable as a function of substrate concentration (apparent K m = 0.762 ± 0.10 μM); (5) inhibited (with similar affinity) by reduced, substituted, and oxidized folate derivatives; and (6) sensitive to the inhibitory effect of anion transport inhibitors. RT-PCR and Western blot analysis showed expression of the human reduced folate carrier (hRFC) at the RNA and protein levels, respectively. The functional contribution of hRFC in carrier-mediated folate uptake was confirmed by gene silencing using gene-specific small interfering RNA. Evidence also was found suggesting that the folate uptake process by MIA PaCa-2 cells is regulated by cAMP- and protein tyrosine kinase (PTK)-mediated pathways. These studies demonstrate for the first time the involvement of a specialized, acidic pH-dependent, carrier-mediated mechanism for folate uptake by human pancreatic MIA PaCa-2 cells. The results also show the involvement of hRFC in the uptake process and suggest the possible involvement of intracellular cAMP- and PTK-mediated pathways in the regulation of folate uptake.

human reduced folate carrier; small interfering RNA; transport regulation

FOLATE, a WATER-SOLUBLE VITAMIN, is essential for normal cellular functions, growth, and development. It acts as a cofactor in various one-carbon (methyl group) transfer reactions that are involved in the synthesis of precursors of DNA and RNA as well as in the biosynthesis of certain amino acids. For this reason, cellular deficiency of this essential micronutrient leads to a disturbance in the normal physiology of the cell that ultimately manifests itself in the form of undesirable clinical symptoms. All human cells cannot synthesize folate, and thus the vitamin must be obtained from the surrounding environment. Previous studies characterized the cellular and molecular mechanisms involved in folate uptake by a variety of tissues of the digestive system and shed light on their regulation (6, 11, 17). These studies have shown that the reduced folate carrier (RFC) plays a predominant role in folate uptake by tissues such as the intestine and the liver (6, 11, 17). In contrast to the understanding of the mechanism of folate uptake by such digestive tissues, nothing is currently known about the mechanism and regulation of folate uptake by cells of the exocrine pancreas. The pancreas is second only to the liver in terms of its folate content, and folate deficiency leads to a significant impairment in its function (1, 2). A reduction in amylase secretion, the appearance of immature secretory granules in pancreatic cells, and the disappearance of secreted materials in the pancreatic duct have been observed in rats rendered folate deficient (1, 2). Other studies have suggested that disturbances in one-carbon (methyl group) metabolism in the pancreas may contribute to the pathogenesis of several pancreatic disorders (reviewed in Ref. 8) and that a significant inverse relationship exists between serum folate level and risk of human pancreatic cancer (16). Thus studies of the mechanism and regulation of folate uptake by cells of the exocrine pancreas are of physiological, nutritional, and clinical importance and may lead to the design of effective strategies to optimize cellular folate homeostasis in this important organ. For these reasons, we initiated a series of investigations to characterize the mechanism and regulation of folate uptake by cells of the pancreas, and in this report we describe our findings regarding folate uptake by human-derived pancreatic MIA PaCa-2 cells. Our results indicate the existence of a specialized, acidic pH-dependent, carrier-mediated mechanism for folate uptake by these cells. They also show the involvement of human RFC (hRFC) and provide evidence for possible involvement of intracellular cAMP- and protein tyrosine kinase (PTK)-mediated pathways in the regulation of the folate uptake process.

MATERIALS AND METHODS

[3H]folic acid (specific activity, 26.2 Ci/mmol; radiochemical purity, 98.0%) was obtained from Moravek Biochemicals (Brea, CA). [3H]biotin (specific activity, 58.2 Ci/mmol; radiochemical purity, 97%) was purchased from DuPont NEN (Boston, MA). The culture medium, trypsin, and other cell culture ingredients were obtained from Sigma (St. Louis, MO).

Cell Culture and Uptake Studies

Human-derived pancreatic carcinoma epithelial MIA PaCa-2 cells (passage 125; American Type Culture Collection, Manassas, VA; these cells were derived from tumor tissue of the pancreas obtained from a 65-year-old Caucasian male) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% (vol/vol) fetal bovine serum in 75-cm² plastic flasks at 37°C in 5% CO₂-95% air
atmosphere, with the medium changed every 2–3 days. MIA PaCa-2 cells were subcultured by trypsinization (subcultivation ratio 1:5) and plated onto 24-well plates. Uptake studies were performed on confluent cell monolayers (between passages 127 and 140) 2–3 days after confluence.

\[^{3}H\]folic acid uptake was examined in cells incubated in Krebs-Ringer buffer containing (in mM) 133 NaCl, 4.93 KCl, 1.23 MgSO\(_4\), 0.85 CaCl\(_2\), 5 glucose, 5 glutamine, 10 HEPES, and 10 MES, pH 5.0 (unless otherwise stated). \[^{3}H\]folic acid was added to the incubation medium at the outset of the uptake experiment, and the reaction was terminated after 3 min (unless otherwise stated) by the addition of 1 ml of ice-cold buffer followed by immediate aspiration. Cells were then rinsed twice with ice-cold buffer and lysed with 1 ml of 1 N NaOH. Lysates were neutralized with HCl, and then radioactivity was measured in a scintillation counter. The protein content of cell digests were measured in parallel wells using a Bio-Rad D\(_2\) protein assay kit (Bio-Rad, Richmond, VA).

**Western Blot Analysis**

Western blot analysis was performed as described previously (4) with the use of anti-hRFC polyclonal antibodies. Briefly, MIA PaCa-2 cells were lysed with 20 mM Tris-HCl, pH 7.4, containing 100 mM KCl, 0.9% Triton X-100, 2 mM phenylmethylsulfonyl fluoride, 1 \(\mu\)g/ml aprotinin, and 0.5 \(\mu\)g/ml leupeptin. Postnuclear extracts (100 \(\mu\)g protein) were subjected to SDS-8% PAGE and electroblotted on Hybond ECL nitrocellulose membrane (Amersham Phamacia Biotech, Piscataway, NJ). After blocking with 5% powdered defatted milk in PBS-Tween 20, blots were incubated with rabbit anti-hRFC polyclonal antibodies. Immunodetection was performed with goat anti-rabbit IgG conjugated to horseeradish peroxidase, using an enhanced chemiluminescence detection system (Amersham, Arlington Heights, IL). Specific bands were quantitated with the use of the Eagle Eye II system (Stratagene, La Jolla, CA).

**Pretreatment with Gene-Specific Small Interfering RNA**

Pretreatment of MIA PaCa-2 cells with hRFC gene-specific RNA was performed as described previously (3). The targeted region for silencing of hRFC (GenBank accession no. NM_003056) was selected from the cDNA sequence beginning 612 nt downstream of the start codon ATG. Custom-made hRFC gene-specific small interfering RNA (siRNA; double-stranded RNA of 21 nucleotides: 5'-aa ggcg ccacgagcaacct dTdT-3') was chemically synthesized by Qigen-Xeragon (Germantown, MD). Both the sense and antisense strands were modified at their 3' ends to increase stability (5). Before the experiments were performed, siRNA duplexes were dissolved in annealing buffer (100 mM potassium acetate, 30 mM HEPES-KOH, 2 mM magnesium acetate, pH 7.4) and reheated to 90°C for 1 min followed by 1 h at 37°C. MIA PaCa-2 cells (40–50% confluent) were transiently transfected with 1 \(\mu\)g siRNA duplex/well of a 24-well plate with the use of Oligofectamine reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. Two types of controls were used to estimate the specificity of the silencing effect: 1) cells transfected with 1 \(\mu\)g/well scrambled siRNA (5'-aa ggcg ccacgagcaacct dTdT-3') and 2) cells pretreated with Oligofectamine reagent. Assays for silencing were performed on confluent monolayers 2–3 days after transfection.

**RT-PCR Analysis**

Oligo(dT) primers and 5 \(\mu\)g of total RNA isolated from MIA PaCa-2 cells were used with a SuperScript RT-PCR kit (Life Technologies, Rockville, MD) to synthesize first-strand cDNA. To amplify the open reading frame of hRFC, we used two gene-specific primers (5'- GCAGGGTGCTCACAACGG-3' and 5'-CAGCATGGGCCCTTCAAAGAATG-3') corresponding to the sequence in the open reading frame of hRFC to produce a 453-bp product. To determine the level of endogenous hRFC in siRNA-pretreated and control cells, we performed PCR within the linear range of amplification. The conditions for semiquantitative PCR were 95°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 1 min (24 cycles). The products were analyzed on 2% agarose gels, the images were captured using an Eagle Eye II system, and the amplified RT-PCR products were normalized to amplified \(\beta\)-actin controls. To confirm the specificity of the siRNA effect on hRFC, we measured the mRNA level of the human thiamin transporter SLC19A2 in siRNA-pretreated and control cells.

**Data Presentation and Statistical Analysis**

Transport data presented in this article are means ± SE of multiple separate uptake determinations and are expressed in picomoles or femtomoles per milligram of protein per time unit. Data were analyzed by performing the Student’s t-test or ANOVA, with statistical significance set at 0.05. Kinetic parameters of saturable folic acid uptake, i.e., maximal velocity (V\(_{\text{max}}\)) and the apparent Michaelis-Menten constant (K\(_{\text{m}}\)) were calculated by using a computerized model of the Michaelis-Menten equation as described by Wilkinson (18). All semiquantitative RT-PCR and Western blot analyses were performed on at least three separate occasions with comparable results. Representative data are presented in this report.

**RESULTS**

**General Characteristics of Folic Acid Uptake by MIA PaCa-2 Cells**

Time course, possible metabolism during transport, and temperature and energy dependency of the uptake process. Uptake of folic acid (11.6 nM) by MIA PaCa-2 cells was examined as a function of time at pH 5.0, was found to be linear for up to 10 min of incubation, and occurred at a rate of 52 fmol·mg protein\(^{-1}\)·min\(^{-1}\) (Fig. 1). Thus we chose a 3-min period as the standard incubation time.

The metabolic form of the transported substrate after the incubation of cells with \[^{3}H\]folic acid was also investigated with the use of cellulose-precoated thin-layer chromatography plates and a solvent system of 0.1 M anhydrous Na\(_2\)HPO\(_4\) at pH 6.8 and 0.5 M potassium acetate, pH 4.8. Fig. 1. Uptake of folic acid by MIA PaCa-2 cells as a function of time. Confluent monolayers of MIA PaCa-2 cells were incubated at 37°C in Krebs-Ringer buffer, pH 5.0, for different time intervals. \[^{3}H\]Folic acid (11.6 nM) was added to the incubation medium at the start of uptake. Each data point represents the mean ± SE of 4–6 separate uptake determinations. When not shown, error bars are smaller than the symbol.
solution, pH 7.0 (12). The results show that 95% of the transported \(^{3}\text{H}\) folic acid was in the intact folate form.

We also examined the effect of incubation temperature on the initial rate of folic acid (11.6 nM) uptake at pH 5.0 and determined the \(Q_{10}\) value (i.e., the ratio of transport rate at 37°C to transport rate at 27°C). The \(Q_{10}\) value was found to be 2.04, which suggests the involvement of a mediated process. In other studies, we examined the effect of pretreating the cells for 30 min with the metabolic inhibitors 2,4-dinitrophenol (DNP) and iodoacetate (both at 1 mM) on the initial rate of folic acid (11.6 nM) uptake at pH 5.0. The results show significant \((P < 0.01)\) inhibition in the substrate uptake by both pretreatments (136.09 ± 0.9, 60.88 ± 1.5, and 82.79 ± 2.2 fmol·mg protein\(^{-1}\)·3 min\(^{-1}\) for control and in cells pretreated with DNP and iodoacetate, respectively).

**Effect of Na\(^{+}\) and incubation buffer pH.** We sought to determine whether the uptake of folic acid into MIA PaCa-2 cells depends on the availability of Na\(^{+}\) in the incubation medium. We examined the effect of isosmotically replacing Na\(^{+}\) (123 mM) in the incubation medium with the monovalent cations K\(^{+}\) and Li\(^{+}\) (123 mM) on the initial rate of folic acid (11.6 nM) uptake at pH 5.0. The results show that replacing Na\(^{+}\) with either K\(^{+}\) or Li\(^{+}\) had no significant effect on vitamin uptake [148.3 ± 7.4, 148.7 ± 2.9, and 146.4 ± 2.5 fmol·mg protein\(^{-1}\)·3 min\(^{-1}\) for control (Na\(^{+}\)) and in the presence of K\(^{+}\) and Li\(^{+}\), respectively]. We also examined the effect of pretreating MIA PaCa-2 cells for 30 min with the Na\(^{+}\)-K\(^{+}\)-ATPase inhibitor ouabain (1 mM) on the initial rate of folic acid (11.6 nM) uptake at pH 5.0. The results show similar uptake in ouabain-pretreated and control cells (131.00 ± 3.3 and 136.09 ± 0.9 fmol·mg protein\(^{-1}\)·3 min\(^{-1}\), respectively).

In another study, we examined the effect of varying incubation buffer pH over the range 5.0 to 8.0 on the initial rate of folic acid (11.6 nM) uptake by MIA PaCa-2 cells. The results (Fig. 2) show that decreasing the incubation buffer pH from 8.0 to 5.0 caused a sharp increase in folic acid uptake with maximum uptake at pH 5.0. In fact, folic acid uptake at pH 7.4 was only ~2% of that at pH 5.0. For this reason, we performed all other experiments at buffer pH 5.0. In a related study, we tested the effect of pretreating for 30 min MIA PaCa-2 cells with the protonophore carbonyl cyanide \(p\)-trifluoromethoxy-phenylhydrazone (FCCP; 50 \(\mu\)M) on the initial rate of folic acid (11.6 nM) uptake at pH 5.0 and 7.4. FCCP caused significant \((P < 0.01)\) inhibition in folic acid uptake in cells incubated at buffer pH 5.0 (137.42 ± 6.4 and 77.4 ± 1.5 fmol·mg protein\(^{-1}\)·3 min\(^{-1}\) for control and FCCP-pretreated cells, respectively) but not in those incubated at buffer pH 7.4 (3.08 ± 0.5 and 3.24 ± 0.5 fmol·mg protein\(^{-1}\)·3 min\(^{-1}\) for control and FCCP-pretreated cells, respectively).

**Evidence for Existence of Carrier-Mediated Mechanism for Folic Acid Uptake by MIA PaCa-2 Cells**

**Uptake as a function of concentration.** In this study, we examined the initial rate of folic acid uptake (3 min) as a function of increasing the substrate concentration in the incubation medium (0.02–10 \(\mu\)M). The results (Fig. 3) show that the uptake exhibited saturation with respect to increasing the folic acid concentration in the medium. Uptake by the saturable component was determined by subtracting the uptake by simple diffusion from the total folic acid uptake at each substrate concentration examined. Uptake by simple diffusion was calculated as the slope of the line between uptake at high pharmacological concentration of folic acid (1 mM) and the point of origin. The apparent \(K_{m}\) and \(V_{max}\) for the saturable uptake component were then calculated as described in MATERIALS AND METHODS and found to be 0.762 ± 0.10 \(\mu\)M and 6.115 ± 0.29 fmol·mg protein\(^{-1}\)·3 min\(^{-1}\), respectively.

**Effect of folic acid structural analogs on uptake of \(^{3}\text{H}\) folic acid.** In this investigation, we tested the effect of the different concentrations of the folic acid structural analogs 5-formyltetrahydrofolate (5-FTHF) and methotrexate (MTX) on the initial rate of \(^{3}\text{H}\) folic acid (11.6 nM) uptake by MIA PaCa-2 cells. The results indicate that both analogs inhibited the uptake of \(^{3}\text{H}\) folic acid and that the inhibition was competitive in nature, with inhibition constants \((K_{i})\) of 1.0 and 0.83 \(\mu\)M for 5-FTHF and MTX, respectively.

**Effect of membrane transport inhibitors.** We examined the effect of inhibitors of anion exchangers, i.e., 4,4’-diiidothyroanostilbene-2,2’-disulfonic acid (DIDS) and 4-acetamido-4’-isothiocyanostilbene-2,2’-disulfonic acid (SITS) (both at 0.5 mM), on the initial rate of folic acid (11.6 nM) uptake by MIA PaCa-2 cells. Both membrane transport inhibitors significantly \((P < 0.01)\) inhibited the folic acid uptake (150.2 ± 3.7, 3.6 ± 0.6, and 15.0 ± 0.8 fmol·mg protein\(^{-1}\)·3 min\(^{-1}\) for control and in cells pretreated with DIDS and SITS, respectively).

**Expression of hRFC at RNA and Protein Levels in MIA PaCa-2 Cells and Evidence for Functional Contribution of hRFC**

The findings that the folic acid uptake process by MIA PaCa-2 cells is markedly higher at acidic pH compared with alkaline pH, that the process has a similar affinity for oxidized, reduced, and substituted folate derivatives, and that it has an apparent \(K_{m}\) in the micromolar range strongly suggest that the process is mediated via hRFC (11–14). Thus our next aim was to establish the expression of the hRFC at the mRNA and...
protein levels in MIA PaCa-2 cells and to further demonstrate its functional contribution to carrier-mediated folate uptake. We performed RT-PCR with the use of gene-specific primers corresponding to a sequence in the open reading frame of hRFC (see MATERIALS AND METHODS) and obtained the fragment of expected size (Fig. 4A). Sequencing of the fragment showed that it was identical to that of hRFC. Expression of hRFC in these cells at the protein level was confirmed by Western blot analysis with the use of specific anti-hRFC polyclonal antibodies and postnuclear extracts of MIA PaCa-2 cells (Fig. 4B).

To determine the functional contribution of hRFC to carrier-mediated folate uptake by MIA PaCa-2 cells, we examined the effect of silencing the hRFC gene by means of siRNA on the ability of the cells to transport the vitamin. First, we established the mechanism of folic acid uptake by MIA PaCa-2 cells, we examined possible regulation of the uptake process by specific intracellular regulatory pathways. We focused on pathways for which there is a consensus sequence in the hRFC protein (i.e., PKC and PKA) (9, 10) and on those that have been shown to play a role in the regulation of uptake of other nutrients in other cellular systems (PTK- and Ca2+/calmodulin-mediated pathways; see Ref. 7).

Involvement of a PKC-mediated pathway in the regulation of folic acid uptake by MIA PaCa-2 cells was examined by pretreating (for 1 h) confluent monolayers with modulators of PKC activity before uptake measurements. We tested the effect of pretreating the cells for 1 h with compounds that are known to increase intracellular cAMP level, namely, dibutyryl cAMP (DBcAMP) and isobutylmethylxanthine (IBMX), on the initial rate of folic acid (11.6 nM) uptake. None of these modulators were found to significantly affect folic acid uptake (106.7 ± 2.8, 98.2 ± 7.9, 108.3 ± 2.2, and 98.1 ± 6.0 fmol·mg protein−1·min−1 for control cells and in cells pretreated with PMA, chelerythrine, and bisindolylmaleimide, respectively).

The role of a cAMP (or PKA)-mediated pathway in the regulation of folic acid uptake by MIA PaCa-2 cells was investigated by examining the effect of pretreating the cells for 1 h with compounds that are known to increase intracellular cAMP level, namely, dibutyryl cAMP (DBcAMP) and isobutylmethylxanthine (IBMX), on the initial rate of folic acid (11.6 nM) uptake. DBcAMP and IBMX were found to cause a
We then examined whether the effect of genistein was mediated by the increase in the apparent protein kinase A activity on [3H]folic acid uptake by MIA PaCa-2 cells. This was accomplished by studying the effect of DBcAMP (2 mM) on the initial rate of folic acid uptake as a function of concentration and comparing the results with that of untreated control. The results show carrier-mediated folic acid uptake to be saturable in both the absence and the presence of DBcAMP but that uptake in the presence of DBcAMP was lower than that of control (Fig. 6). The kinetic parameters of saturable uptake showed a decrease in the V_{max} of folic acid uptake in DBcAMP-pretreated cells compared with control cells (2.418 ± 0.07 and 6.115 ± 0.29 fmol·mg protein^{-1}·min^{-1}, respectively), as well as a decrease in the apparent K_{m} (0.358 ± 0.03 and 0.762 ± 0.10 μM, respectively).

A possible role for PTK in the regulation of folic acid uptake by MIA PaCa-2 cells was also tested by examining the effect of pretreating cells for 1 h at 37°C with the PTK inhibitors genistein and tyrphostin A25 on the initial rate of folic acid uptake (Table 1). Pretreating the cells with the PKA inhibitor H-89 failed to cause any significant decrease in the uptake of folic acid and that causes a significant and concentration-dependent inhibition in folic acid uptake (Table 1). Pretreating the cells with the PTK inhibitor H-89 failed to cause any significant effect on folic acid uptake. In addition, pretreating cells with H-89 (100 μM) first for 30 min, followed by the addition of 1 mM DBcAMP and subsequent incubation for 30 min, failed to prevent the inhibition in folic acid uptake caused by DBcAMP alone (112.2 ± 2.6, 67.6 ± 2.0, and 64.0 ± 0.7 fmol·mg protein^{-1}·min^{-1} for the control, DBcAMP-treated, and both DBcAMP- and H-89-treated cells, respectively). We also examined the effect of DBcAMP on the kinetic parameters of the folic acid uptake process by MIA PaCa-2 cells. This was accomplished by examining the effect of DBcAMP (2 mM) on the initial rate of folic acid uptake by MIA PaCa-2 cells. This was accomplished by examining the effect of pretreating the cells for 1 h with the compound under investigation. [3H]folic acid (11.6 nM) was then added, and incubation was continued at 37°C for 3 min at pH 5.0. Data are means ± SE of 4–10 separate uptake determinations. DBcAMP, dibutyryl cAMP; IBMX, isobutyl methylxanthine; NS, not significant.

![Graph](http://ajpcell.physiology.org/Downloadedfrom/http://ajpcell.physiology.org/)

**Fig. 6.** Effect of dibutyryl cAMP (DBcAMP) on uptake of folic acid by MIA PaCa-2 cells as a function of folic acid concentration. Confluent monolayers of MIA PaCa-2 cells were pretreated for 1 h with (●, 2 mM) or without (○) DBcAMP. Uptake of different concentrations of folic acid (0.02–10 μM) was then examined after 3-min incubation (i.e., initial rate) at 37°C in Krebs-Ringer buffer, pH 5.0. Uptake by the carrier-mediated process was determined as described in the text. Kinetic parameters of the saturable process were determined as described in MATERIALS AND METHODS. Values are means ± SE of 4–6 separate uptake determinations. When not shown, error bars are smaller than the symbol.

### DISCUSSION

Our results show that folic acid uptake by MIA PaCa-2 cells is temperature and energy dependent and proceeds without metabolic alterations in the transported substrate. Uptake was also found to be Na⁺ independent, as indicated by the lack of increased with such treatment (1.403 ± 0.19 and 0.762 ± 0.10 μM, respectively).

We also investigated the possible involvement of the Ca^{2+}/calmodulin-mediated pathways in the regulation of folic acid uptake by MIA PaCa-2 cells. This was accomplished by examining the effect of pretreating the cells (for 1 h) with inhibitors of this pathway, namely, trifluoperazine, calmidazolium, and W13 (all at 50 μM), on the initial rate of uptake of 11.6 nM folic acid. The results show that none of these compounds had a significant effect on folic acid uptake (106.2 ± 4.3, 105.2 ± 3.2, 108.3 ± 4.6, and 109.9 ± 5.0 fmol·mg protein^{-1}·min^{-1} for control and trifluoperazine-, W13-, and calmidazolium-pretreated cells, respectively).

### Table 1. Effect of modulators of intracellular cAMP level and protein kinase A activity on [3H]folic acid uptake by MIA PaCa-2 cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>Concentration</th>
<th>Uptake, fmol·mg protein^{-1}·min^{-1}</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>112.3±2.4</td>
<td></td>
</tr>
<tr>
<td>DBcAMP</td>
<td>1 mM</td>
<td>56.4±3.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>5 mM</td>
<td>21.6±0.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>IBMX</td>
<td>2.5 mM</td>
<td>55.8±5.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>5 mM</td>
<td>34.3±3.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>H-89</td>
<td>50 μM</td>
<td>115.0±8.2</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>100 μM</td>
<td>115.1±4.2</td>
<td>NS</td>
</tr>
</tbody>
</table>

Confluent monolayers of MIA PaCa-2 cells were preincubated at 37°C for 1 h with the compound under investigation. [3H]folic acid (11.6 nM) was then added, and incubation was continued at 37°C for 3 min at pH 5.0. Data are means ± SE of 4–10 separate uptake determinations. DBcAMP, dibutyryl cAMP; IBMX, isobutyl methylxanthine; NS, not significant.

### Table 2. Effect of protein tyrosine kinase inhibitors on [3H]folic acid uptake by MIA PaCa-2 cells

<table>
<thead>
<tr>
<th>Condition</th>
<th>Concentration</th>
<th>Uptake, fmol·mg protein^{-1}·min^{-1}</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>108.7±2.5</td>
<td></td>
</tr>
<tr>
<td>Genistein</td>
<td>25 μM</td>
<td>72.2±3.6</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>50 μM</td>
<td>55.7±2.1</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Genistein</td>
<td>100 μM</td>
<td>41.2±2.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Tyrphostin A25</td>
<td>50 μM</td>
<td>72.0±4.5</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Confluent monolayers of MIA PaCa-2 cells were preincubated at 37°C for 1 h with the compound under investigation. [3H]folic acid (11.6 nM) was then added, and incubation was continued at 37°C for 3 min at pH 5.0. Data are means ± SE of 4–10 separate uptake determinations. NS, not significant.
effect of Na\(^+\) removal from the incubation medium on the initial rate of folic acid uptake, as well as by the inability of the Na\(^+\)-K\(^+\)-ATPase inhibitor ouabain to affect folate uptake significantly. In contrast, uptake of folic acid was found to be highly pH dependent and increased as a function of decreasing buffer pH from 7.4 to 5.0. In fact, folic acid uptake at pH 7.4 was found to be merely 2% of that at pH 5.0. In addition, pretreatment of cells with the protonophore FCCP leads to inhibition in the initial rate of folic acid uptake by cells incubated at buffer pH 5 but not by those incubated at pH 7.4. These findings clearly demonstrate the importance of an inwardly directed H\(^+\) gradient in the uptake of the anionic folic acid by these pancreas-derived cells. Whether this is an indication for the involvement of a folate /OH\(^-\) exchange or a folate /-H\(^+\) cotransport mechanism similar to that suggested for liver cells (6) (the liver also accumulates high levels of folate) as well as intestinal epithelial cells (for review, see Ref. 11) requires further investigation.

Folic acid uptake by pancreatic MIA PaCa-2 cells was found to be carrier mediated in nature. This conclusion is based on the observation that the initial rate of folic acid uptake was saturable as a function of substrate concentration, with an apparent \(K_m\) value of 0.762 ± 0.10 \(\mu\)M. The ability of the folate structural analogs 5-FTHF and MTX to inhibit the initial rate of \(^{[3]}\)H\)folic acid uptake further confirms this conclusion. It is interesting to note that the \(K_i\) of \(^{[3]}\)H\)folic acid uptake by 5-FTHF and MTX is competitive in nature, with apparent \(K_m\) of 1.0 and 0.83 \(\mu\)M, respectively. The similarity of the \(K_i\) values for 5-FTHF and MTX and the value of the apparent \(K_m\) for folic acid uptake by MIA PaCa-2 cells strongly suggests that these three compounds have the same affinity for the involved uptake system and thus clearly justifies the use of folic acid as a representative folate compound in this study. It is interesting that the pH profile and the similar affinity for oxidized, reduced, and substituted folate derivatives are similar to those observed in human intestinal and colonic epithelial cells (7, 11–13). These features of the folate uptake process are in contrast to the features observed in cells such as L1210 cells, however, in which uptake is higher at neutral and alkaline than at acidic buffer pH and in which the system has higher affinity for reduced over oxidized folate derivatives (for review, see Ref. 15).

The finding of greater folate uptake by MIA PaCa-2 cells at acidic pH than at alkaline pH, the similar affinity for oxidized, reduced, and substituted folate derivatives, and the fact that the apparent \(K_m\) of the uptake system is in the micromolar range strongly indicate that this system is the hRFC (11–14). RT-PCR and Western blot analysis results confirmed the expression of hRFC at the RNA and protein levels, respectively. To further confirm the functional involvement of the hRFC in folate uptake by MIA PaCa-2 cells, we examined the effect of silencing the hRFC gene on the ability of the cells to take up folic acid by the carrier-mediated process. This experiment was performed with the use of gene-specific siRNA. First, we validated that such an approach does indeed lead to specific depletion in the level of hRFC mRNA and the protein level in the pretreated cells compared with controls. The results show that pretreating the MIA PaCa-2 cells with hRFC gene-specific siRNA leads to a specific and marked decrease in hRFC mRNA and protein levels. We then examined the effect of such siRNA pretreatment on the ability of the cells to transport folic acid. The results showed significantly less folic acid uptake in siRNA-pretreated cells than in control cells. This effect was found to be specific for folic acid because uptake of the unrelated biotin was similar in hRFC siRNA-pretreated and control cells. These findings clearly confirm the functional role of hRFC in folic acid uptake by MIA PaCa-2 cells.

After the determination of the cellular mechanism of folate uptake by MIA PaCa-2 cells, we investigated the possible involvement of certain intracellular protein kinase-mediated regulatory pathways in the regulation of folic acid uptake by these pancreas-derived cells. We focused on those pathways for which there is consensus sequence in the hRFC polypeptide (PKC and PKA; see Refs. 9, 10) and on those that have been shown to regulate the uptake of other nutrients in other cell systems. Our results show that although PKC- and Ca\(^{2+}\)/calmodulin-mediated pathways have no role in the regulation of folic acid uptake by MIA PaCa-2 cells (as indicated by lack of effect on vitamin uptake by modulators of these pathways), evidence was obtained that suggests the involvement of cAMP- and PTK-mediated pathways in the regulation of the uptake process. The effect of cAMP was found to be mediated via changes in the \(V_{\max}\) and the apparent \(K_m\) of the folic acid uptake process, suggesting an effect on the activity and affinity of the folate carrier system, respectively. The effect of increasing the level of intracellular cAMP on folic acid uptake did not appear to be mediated by the activation of PKA. This conclusion is based on the observation that the addition of the PKA-specific inhibitor H-89 failed to prevent the inhibitory effect on folic acid uptake caused by subsequent addition of DBCAMP. The inhibitory effect of genistein was mediated via a decrease in the \(V_{\max}\) and an increase in the apparent \(K_m\) of the folic acid uptake process, suggesting that the effect is mediated via alterations in the activity (and/or number) and the affinity...
of the folic acid uptake carriers. These results regarding the possible role of cAMP and PTK in the regulation of folic acid uptake by MIA PaCa-2 cells are similar to those observed for substrate uptake by other cells of the digestive system (namely, the human colonocytes) (7).

In summary, the results of our study demonstrate for the first time that folate uptake by human-derived pancreatic MIA PaCa-2 cells is mediated via a specialized, acidic pH-dependent, carrier-mediated mechanism that involves the hRFC. In addition, the study shows the folate uptake process of these cells to be under the possible regulation of intracellular cAMP- and PTK-mediated pathways.

GRANTS
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