Available online at www.sciencedirect.com

SCIENCE DIRECT®

Biochimica et Biophysica Acta 1611 (2003) 249–257

BIOCHIMICA ET BIOPHYSICA ACTA

BBA

www.bba-direct.com

Sensitivity of bile acid transport by organic anion-transporting polypeptides to intracellular pH☆

Jose J.G. Marin[a,*], Daniel Mangas[a], Maria C. Martinez-Diez[a], Mohamad Y. El-Mir[a], Oscar Briz[a], Maria A. Serrano[b]

[a] *Department of Physiology and Pharmacology, Faculty of Pharmacy, University of Salamanca, Campus Miguel Unamuno, EID-S09, 37007-Salamanca, Spain*

[b] *Department of Biochemistry and Molecular Biology, University of Salamanca, Salamanca, Spain*

Received 8 January 2003; received in revised form 10 February 2003; accepted 21 February 2003

Abstract

We investigated the influence of intracellular pH (pHi) on [^{14}C]-glycocholate (GC) uptake by human hepatoblastoma HepG2 cells that express sodium-independent (mainly OATP-A and OATP-8), but not sodium-dependent, GC transporters. Replacement of extracellular sodium by choline (Chol) stimulated GC uptake but did not affect GC efflux from loaded cells. Amiloride or NaCl replacement by tetraethylammonium chloride (TeACl) or sucrose also increased GC uptake. All stimulating circumstances decreased pHi. By contrast, adding to the medium ammonium or imidazole, which increased pHi, had no effect on GC uptake. In Chinese hamster ovary (CHO) cells expressing rat Oatp1, acidification of pHi had the opposite effect on GC uptake, that is, this was reduced. Changes in extracellular pH (pHo) between 7.40 and 7.00 had no effect on GC uptake at pHi 7.30 or 7.45 when pHo < pHi. However, GC uptake was inhibited at pHo 7.40 and 7.80 when pHo>pHi. Inhibition was not proportional to the pHo − pHi difference. Intracellular acidification decreased V_{max}, but had no effect on K_m. In sum, sodium-independent GC transport can be affected by intracellular acidification, possibly due both to modifications in the driving forces and to the particular response to protonation of carrier proteins involved in this process.

Keywords: Carrier; Glycocholic acid; Liver; Membrane; Transport

1. Introduction

In mammals, several types of epithelial cells are able to perform carrier-mediated uptake of bile acids, namely, those located in organs forming part of the so-called enterohepatic circulation, such as hepatocytes, cholangiocytes and ileocytes [1]. The mechanisms involved in such uptake include sodium-dependent and sodium-independent processes. In humans, members of the *SLC10A* gene family (symbols in italic are from the Human Gene Nomenclature Committee Data Base), such as sodium-taurocholate cotransporting polypeptide (NTCP, *SLC10A1*) and ileal bile acid transporter (IBAT, *SLC10A2*) are responsible for bile acid uptake driven by sodium gradients, whereas some members of the family of organic anion-transporting polypeptides (OATP, *SCL21A*) seem to account for most of the sodium-independent bile acid uptake in different epithelia [1].

In spite of the important and rapid advances made during the last decade in our knowledge of the molecular biology of the prolific family of OATPs [2], the mechanisms involved in their functional regulation under normal and pathological situations are poorly understood.

Apart from the physiological relevance of these carriers, they are also of pharmacological interest due to the selective partial or complete disappearance of sodium-dependent bile

Abbreviations: BSEP, bile salt export pump; CHO cells, Chinese hamster ovary cells; Chol, choline; DMO, 5,5-dimethyl-[2-^{14}C],4-oxazolidinedione; GC, sodium glycocholate; MRP, multidrug resistance-associated protein; NTCP, sodium-taurocholate co-transporting polypeptide; OATP, multispecific organic anion-transporting polypeptide; TeA, tetraethylammonium

☆ This study was supported in part by Fondo de Investigaciones Sanitarias, Ministerio de Sanidad y Consumo (Grant FIS Ref 01/1043), the Junta de Castilla y León (Grants SA023/02 and DGESIC, Ministerio de Ciencia y Tecnología (PB98-0259), Spain. Dr. Oscar Briz received a Research Fellowship from the Fundacion Miguel Casado San Jose, Salamanca, Spain. The group is member of the Spanish Network for Cooperative Research on Hepatitis (Grant G03/015), Instituto de Salud Carlos III, Spain.

* Corresponding author. Tel.: +34-923-294674; fax: +34-923-294669.
E-mail address: jjgmarin@usal.es (J.J.G. Marin).

0005-2736/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S0005-2736(03)00080-4

acid transport activity in de-differentiated parenchymal liver cells, which in contrast express OATPs [3–5]. This has inspired several investigations aimed at using sodium-independent bile acid carriers to target cytostatic drugs toward liver tumors [6]. An explanation for the selective effect of de-differentiated hepatocytes and the level of expression between sodium-dependent and -independent bile acid transporters can be found in the ontogenic and phylogenic patterns of these carriers. Sodium-dependent bile acid carriers probably appeared late in evolution, as suggested by the fact that in lower vertebrates, such as the little skate, rainbow trout, frog, turtle or chicken, orthologues of *SLC10A* gene have not been found and hence the uptake of cholephilic organic anions by hepatocytes in these species must be mediated only by sodium-independent mechanisms [3,7,8]. Sodium-independent bile acid uptake also appears earlier in ontogeny. From studies using basolateral plasma membrane vesicles from fetal rat liver, functional evidence for sodium-independent bile acid transport activity preceding that for sodium-dependent mechanisms has been found [9]. This is consistent with molecular biology studies reporting that the gene transcription of rat Oatp1(*Slc21a1*) in the fetal liver precedes that of Ntcp (*Slc10a1*) [3,10]. Similarly, the intestinal sodium-dependent bile acid transport system also appears late in ontogenic development [11,12].

All the available information suggests that some OATPs may work as anion exchangers. Glutathione has been suggested to be the molecule physiologically exchanged, at least by some of the OATP isoforms [13]. Bicarbonate may also play a role as an exported anion in exchange with the organic anions taken up by rat Oatp1 [14], which could explain in part the sensitivity to changes in pH of cholate uptake by basolateral plasma membrane vesicles obtained from rat liver [15]. In addition to the bicarbonate concentration gradient acting as a driving force for some of these carriers, intracellular pH (pHi) may affect the protonation state of amino acids belonging to cytoplasmic domains of the carrier and hence modify their overall behavior. Indeed, other membrane proteins such as those involved in chloride channels have recently been found to be sensitive to changes in pHi, which can profoundly affect trans-epithelial transport physiology [16]. Therefore, we wondered whether pHi may also be involved in modulating the activity of the carriers responsible for bile acid uptake by liver cells, which may have important functional repercussions in pathological situations in which the control of pHi is impaired.

Many studies have been carried out on HepG2 cell line, which was derived from a biopsied well-differentiated hepatoblastoma obtained by extended lobectomy of a Argentinian 15-year-old Caucasian male [17], because it retains a number of typical hepatocyte functions, including the ability to synthesize [18] and take up [4] bile acids. Although such cells are able to carry out sodium-dependent transport of other substrates such as amino acids [19], regarding bile acid transport, they offer an excellent model for the purpose of the present study, because this process is entirely sodium-independent. This is due to the absence of NTCP expression in these cells, whereas they express OATP-A and OATP-8, but not OATP-C [4,13]. Interestingly, several groups have reported that bile acid uptake by these cells is enhanced if sodium is removed from the incubation medium [4,13]. Similar observations by our group prompted us to further investigate the reason for this finding. Because our preliminary results had indicated that an effect of changes in pHi on sodium-independent bile acid uptake seemed to be involved, the study was extended to evaluate the effect of changes in pHi on bile acid transport by a different sodium-independent carrier, rat Oatp1. We used Chinese hamster ovary (CHO) cells stably transfected with the cDNA of rat Oatp1 as a representative member of the Oatp family in this species. We used this model because the uptake of organic solutes by rat Oatp1 has been reported to be carried out by a mechanism independent of either ATP hydrolysis or the trans-membrane gradient of H^+, Na^+, K^+, or Cl^- [20,21], which was expected to simplify the interpretation of our results.

2. Materials and methods

2.1. Chemicals and cells

[^{14}C]-glycocholic acid (GC; specific activity: 56 mCi/mmol) was from Amersham Pharmacia Biotech (Barcelona, Spain), [^{3}H]-inulin (specific activity: 304.8 mCi/g) and [^{3}H]-H_2O (specific activity: 1 mCi/g) were from Perkin-Elmer Life Science (Pacisa and Giralt, Madrid, Spain). [^{14}C]-5,5-dimethyl-[2-^{14}C],4-oxazolidinedione ([^{14}C]-DMO; specific activity: 55 mCi/mmol) was from American Radiolabeled Chemicals Inc. (Itisa, Madrid, Spain). All other chemicals and culture media were from Sigma-Aldrich (Madrid, Spain) or Merck Eurolab (Barcelona, Spain).

Human hepatoblastoma HepG2 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). These cells (2×10^5 cells per 3.5 cm-diameter dishes) were seeded on rat collagen type II and cultured with F-12 Coon's Modification medium supplemented with 26 mM $NaHCO_3$, 5% non-heat-inactivated fetal calf serum and antibiotic cocktail (100 U/ml penicillin, 100 μg/ml streptomycin and 0.25 μg/ml amphotericin B). They were used after 2 days in culture, before confluence was reached. CHO cells, either the wild-type (CHO-K1) or stably transfected with the cDNA of rat Slc21a1 or Oatp1 (CHO 0-3) [22], were generously donated by Drs. Bruno Hagenbuch, Bruno Stieger and Peter Meier (University of Zurich, Switzerland). They were seeded (4×10^5 cells per 3.5 cm-diameter dish) in the absence of rat collagen and were cultured in DMEM (10 g/l) supplemented with 10% fetal calf serum, 10 mM $NaHCO_3$, 2 mM L-glutamine, 0.43 mM L-proline and antibiotic cocktail. They were used after 3 days in culture, before confluence was reached. CHO 0-3 culture medium also contained 400 μg/ml geneticin (G418) (Roche, Barcelona, Spain). Expression of rat Oatp1 in CHO 0-3 cells was enhanced, as previously

reported [23], by adding 5 mM sodium butyrate to culture medium 24 h before carrying out the experiments.

2.2. Quantitative real-time RT-PCR

The abundance of mRNA corresponding to the human liver transporters OATP-A, OATP-C and OATP-8 was determined by real-time quantitative RT-PCR in total RNA from HepG2 cells. In brief, total RNA (~ 30 μg) was isolated from ~ 6×10^6 HepG2 cells using RNAeasy spin columns (Qiagen, Izasa, Barcelona, Spain), treated with DNase I (Roche), measured using the Ribo-Green RNA-Quantitation kit (Molecular Probes, Leiden, The Netherlands), and subjected to reverse transcription using random nonamers and the Superscript II kit (Invitrogen, Madrid, Spain). PCR was performed using Amplitaq Gold polymerase (Perkin-Elmer Applied Biosystems, Madrid, Spain) in an ABI Prism 5700 Sequence Detection System (Perkin-Elmer Applied Biosystems) with the following thermal conditions: a single cycle at 95 °C for 10 min followed by 50 cycles at 95 °C for 15 s and 60 °C for 60 s. Primer oligonucleotides obtained from Sigma-Genosys were designed with the assistance of Primer Express software (Perkin-Elmer Applied Biosystems) for cDNA fragments in described sequences, and their specificity was checked using BLAST (Table 1). Detection was carried out using SYBR Green I. Non-specific products of PCR, as detected by 2.5% agarose gel electrophoresis or melting temperature curves, were not found in any case. DNA used in building standard curves was previously obtained by RT-PCR from human liver (Invitrogen), purified by 2.5% agarose gel electrophoresis, extracted and its amount determined by PicoGreen test (Molecular Probes). Results of mRNA abundance for each target gene in each sample were normalized on the basis of its 18S rRNA content, which was measured with the TaqMan® Ribosomal RNA Control Reagents kit (Perkin-Elmer Applied Biosystems, Madrid, Spain).

2.3. Experimental conditions

Before using the cells to determine GC uptake or pHi, they were transferred to an equilibration medium, EM (96 mM NaCl, 5.3 mM KCl, 1.1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11 mM glucose, 50 mM Hepes, pH 7.40 adjusted using 1 M Trizma® base and 1 N HCl), with which they were incubated at 37 °C for 60 min. To investigate the effect of the absence of sodium in the incubation medium, NaCl was replaced by equiosmolar amounts of choline chloride (CholCl-medium), tetraethylammonium chloride (TeACl-medium) or sucrose. Several strategies were used to modify

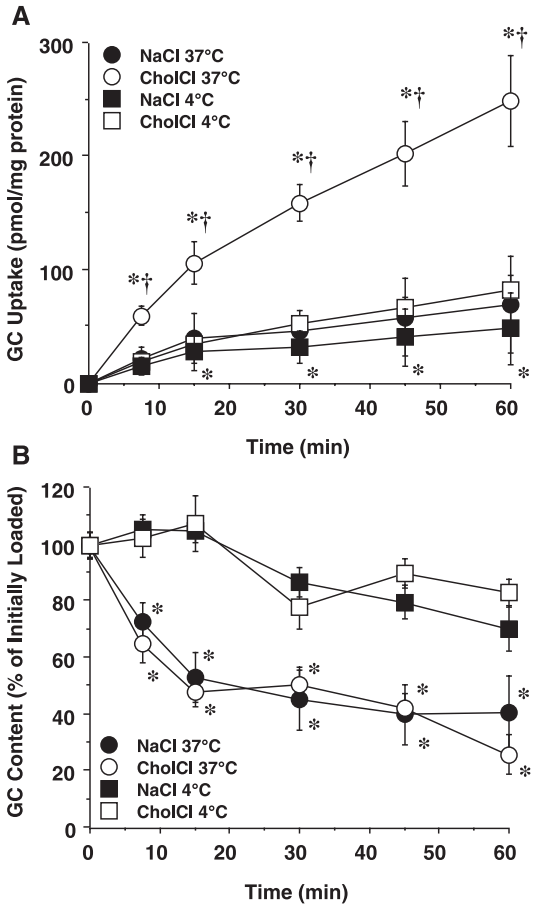

Fig. 1. (A) Time-course of [^{14}C]-glycocholic acid (GC) uptake by HepG2 cells over 60 min. After removal of the culture medium, the cells were equilibrated at 37 °C for 60 min with NaCl-medium (96 mM NaCl, 5.3 mM KCl, 1.1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11 mM glucose, 50 mM Hepes, pH 7.40). Uptake experiments began with the replacement of this medium by another similar one or one in which 96 mM NaCl had been replaced by 96 mM cholineCl (CholCl-medium). In both cases, the medium contained 10 μM GC. Incubations were carried out at 4 or 37 °C. (B) Subsequent GC efflux after loading the cells at 37 °C for 60 min and being transferred to GC-free media. Each data point is the mean ± S.E. from 12 plates corresponding to four cultures in which measurements were performed on three plates per experimental condition and incubation time. *, $P < 0.05$, on comparing 37 °C to 4 °C; †, $P < 0.05$, on comparing CholCl to NaCl by the paired *t*-test.

Table 1
Quantitative real-time RT-PCR

	Forward primer (5′–3′)	Reverse primer (5′–3′)	mRNA level (copies/10^{11} copies of 18S rRNA)
OATP-A	AAGACCAACGCAGGATCCAT	GAGTTTCACCCATTCCACGTACA	748 ± 52
OATP-C	GAATGCCCAAGAGATGATGCTT	AATGCCTGCCAGCGACGAGTATA	3.3 ± 1.2
OATP-8	GTCCAGTCATTGGCTTTGCA	CAACCCAACGAGAGTCCTTAGG	7081 ± 295

Values are means ± S.D. from determinations carried out in triplicate on total RNA obtained from three different cultures of HepG2 cells. The level of 18S rRNA in each sample was used to normalize the results.

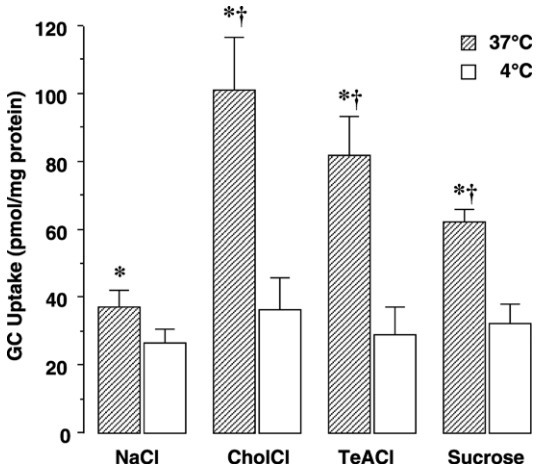

Fig. 2. Effect of sodium replacement on [^{14}C]-glycocholic acid (GC) uptake by HepG2 cells. After removal of the culture medium, the cells were equilibrated at 37 °C for 60 min with NaCl-medium (96 mM NaCl, 5.3 mM KCl, 1.1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11 mM glucose, 50 mM Hepes, pH 7.40). Uptake experiments began with the replacement of this medium by another similar one or one in which 96 mM NaCl had been replaced by 96 mM cholineCl (CholCl-medium), 96 mM tetraethylammonium chloride (TeACl-medium), or 192 mM sucrose (sucrose-medium). In all cases, the medium contained 10 μM GC. Incubations were carried out for 15 min at either 4 or 37 °C. Each data point is the mean ± S.E. of 12 plates corresponding to four cultures in which measurements were performed on three plates per experimental condition and incubation time. *, $P<0.05$, on comparing 37 °C to 4 °C; †, $P<0.05$, as compared to NaCl-medium by the paired *t*-test.

pHi. Amiloride (1 mM) was added to acidify pHi by blocking H^+:Na^+ exchange. In other experiments, 96 mM NaCl in the incubation medium was partially replaced by 30 mM ammonium chloride (NH_4Cl) or 15 mM imidazole (Im). To study the effect of ΔpHi of different magnitudes and directions, both pHi (by including amiloride in NaCl-containing medium) and pHo (by adjusting the pH of the incubation medium with 1 mM Trizma® base or 1 N HCl) were changed.

2.4. Glycocholic acid uptake and efflux

Uptake experiments began with the replacement of EM by a similar one or by another in which some of the variations described above had been included, although always containing 10 μM GC, except in kinetic studies, in which GC concentrations ranged from 5 to 200 μM. In efflux studies, the cells were incubated in the presence of 10 μM GC for 60 min and then transferred to GC-free EM. Once the incubation period had finished either in the uptake or efflux studies, the assay medium was removed by aspiration and the cells were rapidly washed four times with 1.5 ml ice-cold EM. To measure radioactivity and protein, the cells were lysed with 1 ml Lowry medium (100 mM NaOH and 189 mM Na_2CO_3) for at least 2 h at room temperature. In some experiments, the amount of extracellular incubation medium that remained adhering to the cells and culture dish after the washing procedure was calculated using [^{3}H]-inulin as a marker for extracellular water space [24]. Because in all experimental conditions used in this study the amount of GC due to contamination of cells with the incubation medium was < 1%, this value was not taken into account in further calculations.

2.5. Intracellular pH measurements

To determine pHi, the method based on the intracellular/extracellular equilibrium distribution of [^{14}C]-DMO [25] was used as previously described [26]. This method was chosen because of its similarity with the experimental conditions used in uptake experiments. Both 1 mM [^{14}C]-DMO 7×10^5 dpm/ml and 14×10^5 dpm/ml [^{3}H]-H_2O were included in the

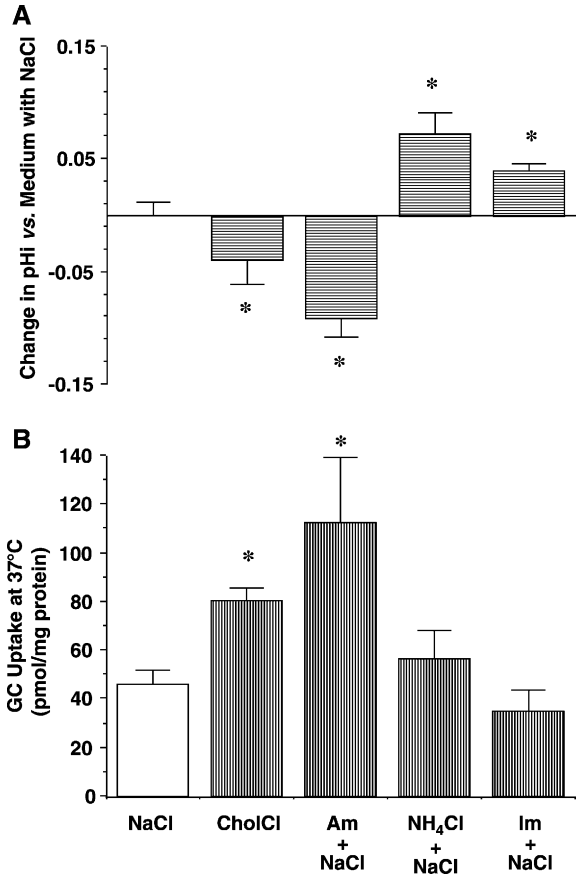

Fig. 3. Change in intracellular pH induced by modification of the incubation medium as compared with the NaCl-medium (A) and effect on [^{14}C]-glycocholic acid (GC) uptake by HepG2 cells (B). After removal of the culture medium, the cells were equilibrated at 37 °C for 60 min with NaCl-medium (96 mM NaCl, 5.3 mM KCl, 1.1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11 mM glucose, 50 mM Hepes, pH 7.40). Uptake experiments began with the replacement of this medium by another similar one or one in which 96 mM NaCl had been replaced completely by 96 mM cholineCl (CholCl-medium), or partially by 1 mM amiloride (Am), 30 mM ammonium chloride (NH_4Cl) or 15 mM imidazole (Im). In all cases, the medium contained either 10 μM GC (uptake measurements) or 1 mM [^{14}C]-dimethyloxazolidine dione (DMO) plus [^{3}H]-H_2O (pHi experiments). Incubations were carried out for 15 min at 37 °C. Each data point is the mean ± S.E. of 12 plates corresponding to four cultures in which measurements were performed on three plates per experimental condition and incubation time. *, $P<0.05$, as compared to the NaCl-medium by the paired *t*-test.

medium 15 min before cell harvesting. At the end of the incubation period, the medium was rapidly collected to measure pHo, using a microprobe, and to determine radioactivity in the incubation medium. The cells were washed and digested as described above for uptake experiments.

2.6. Analytical and statistical methods

Radioactivity was measured by liquid scintillation (Beckman LS-6000, Beckman Instruments, Madrid, Spain) using 4 ml of Universol as scintillant from ICN (Biolink, Barcelona, Spain). Bovine serum albumin was used as standard to measure proteins [27]. Results are expressed as means ± S.E. S.E. To calculate the statistical significance of the differences, the paired *t*-test was used. For the sake of clarity, only a significance level of $P < 0.05$ is indicated. The kinetic parameters for [^{14}C]-GC uptake were calculated after fitting the data with an iterative non-linear least-squares method, using the UltraFit-v2.1 software provided by Biosoft (Cambridge, UK) to the Michaelis–Menten equation for a single carrier system: $V=(V_{max}S)/(K_m+S)$, where V is the initial velocity of uptake; S is the value of the substrate concentration in the incubation medium; K_m is the apparent affinity constant or Michaelis–Menten constant; and V_{max} is the maximal transport velocity.

3. Results

3.1. Studies on HepG2 cells

As compared to normal human liver, the steady-state level of mRNA for all the OATPs assayed was markedly reduced in HepG2. When this was expressed as the percentage of the hepatic level, these values were approximately 0.17% for OATP-A, 0.00002% for OATP-C and 0.012% for OATP-8. Absolute determination of mRNA levels for these carriers in HepG2 cells (Table 1) indicated that OATP-C was almost absent, whereas OATP-8 was approximately 10-fold more abundant than OATP-A. Because both carriers are believed to transport bile acids [2], although not in all expression systems [28], they were considered as the main candidates accounting for GC uptake by these cells. This is consistent with the fact that this process was temperature-sensitive and not stimulated by

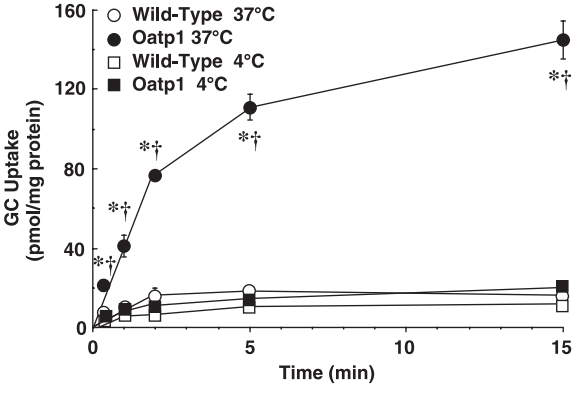

Fig. 4. Time-course of [^{14}C]-glycocholic acid (GC) uptake by wild-type or rat-Oatp1-expressing CHO cells. After removal of the culture medium, the cells were equilibrated at 37 °C for 60 min with NaCl-medium (96 mM NaCl, 5.3 mM KCl, 1.1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11 mM glucose, 50 mM Hepes, pH 7.40). Uptake experiments began with the replacement of this medium by another similar one containing 10 μM GC. Incubations were carried out at 4 or 37 °C. Each data point is the mean ± S.E. from 12 plates corresponding to four cultures in which measurements were performed on three plates per experimental condition and incubation time. *, $P < 0.05$, on comparing 37 °C to 4 °C; †, $P < 0.05$, on comparing wild-type to rat-Oatp1-expressing cells by the paired *t*-test.

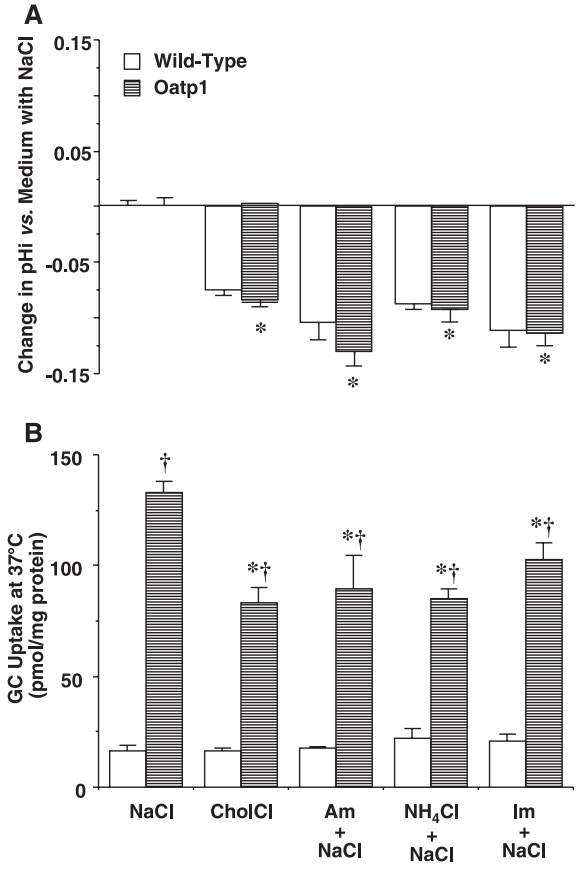

Fig. 5. Change in intracellular pH induced by a modification of the incubation medium as compared to NaCl-medium (A) and effect on [^{14}C]-glycocholic acid (GC) uptake by wild-type or rat-Oatp1-expressing CHO cells (B). After removal of the culture medium, the cells were equilibrated at 37 °C for 60 min with NaCl-medium (96 mM NaCl, 5.3 mM KCl, 1.1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11 mM glucose, 50 mM Hepes, pH 7.40). Uptake experiments began with the replacement of this medium by another similar one or one in which 96 mM NaCl had been replaced completely by 96 mM cholineCl (CholCl-medium), or partially by 1 mM amiloride (Am), 30 mM ammonium chloride (NH_4Cl) or 15 mM imidazole (Im). In all cases, the medium contained either 10 μM GC (uptake measurements) or 1 mM [^{14}C]-dimethyloxazolidine dione (DMO) plus [^{3}H]-H_2O (pHi experiments). Incubations were carried out for 15 min at 37 °C. Each data point is the mean ± S.E. of 12 plates corresponding to four cultures in which measurements were performed on three plates per experimental condition and incubation time. *, $P < 0.05$, as compared to NaCl-medium; †, $P < 0.05$, on comparing wild-type to rat-Oatp1-expressing cells by the paired *t*-test.

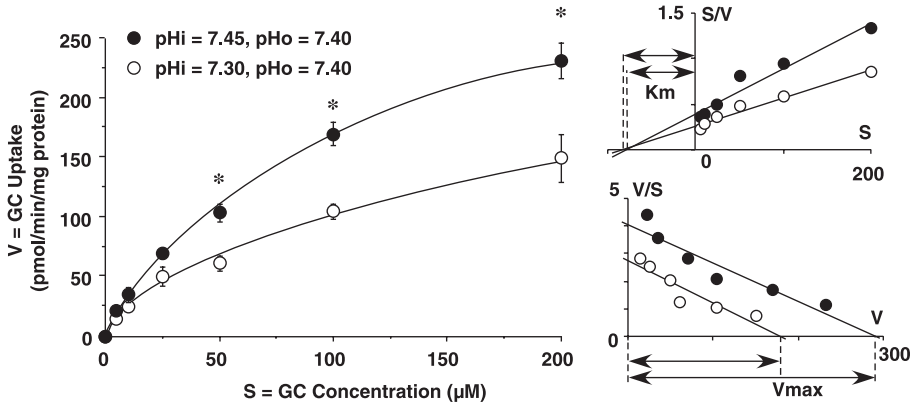

Fig. 6. Effect of change in intracellular pH (pHi) on the rate of uptake (V) of [^{14}C]-glycocholic acid (GC) by rat-Oatp1-expressing CHO cells versus substrate concentrations (S). After removal of the culture medium, the cells were equilibrated at 37 °C for 60 min with NaCl-medium (96 mM NaCl, 5.3 mM KCl, 1.1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11 mM glucose, 50 mM Hepes, pH 7.40). Intracellular pH was not modified (pHi = 7.44 ± 0.04; i.e. approximately 7.45) or was reduced (pHi = 7.32 ± 0.03; i.e. approximately 7.30) by the addition of 1 mM amiloride (Am) 15 min immediately before the uptake period. This began with the replacement of the incubation medium by another similar one containing 5–200 μM GC (uptake measurements). Incubations were carried out for 60 s at 37 °C. To measure pHi, in separate plates, 1 mM [^{14}C]-dimethyloxazolidine dione (DMO) plus [^{3}H]-H_2O (pHi experiments) were added at min 45 of the equilibration period. Each data point is the mean ± S.E. of 12 plates corresponding to four cultures in which measurements were performed on three plates per experimental condition and incubation time. *, $P < 0.05$, on comparing uptake values at pHi = 7.45 and at pHi = 7.30, by the paired *t*-test. Values are also plotted in the right panels as S/V versus S and V/S versus V.

extracellular sodium (Fig. 1A). By contrast, the replacement of sodium by Chol significantly enhanced GC uptake at 37 °C, but not at 4 °C. GC efflux from previously loaded cells was also sensitive to temperature but was unaffected by sodium replacement (Fig. 1B). To investigate whether the presence of Chol or the absence of sodium was responsible for the observed stimulation in GC uptake, these were replaced by another cation—TeA—or by a neutral compound—sucrose—(Fig. 2). In both cases, GC uptake was higher (more with TeA than with sucrose) than in the presence of sodium. When pHi was measured, replacement of sodium by Chol was found to induce a relative intracellular acidification (Fig. 3A). Blockade of Na^+:H^+ exchange by incubation with amiloride [29] also induced both a decrease in pHi (Fig. 3A) and enhanced GC uptake (Fig. 3B). Imitating other studies in different systems, pHi manipulation was attempted by partially replacing sodium either by NH_4^+ [30] or by imidazole [31]. In HepG2 cells, these manoeuvres caused a significant increase in pHi (Fig. 3A), but did not markedly affect GC uptake (Fig. 3B).

3.2. Studies on CHO cells expressing rat Oatp1

Wild-type CHO cells were not able to carry out efficient GC uptake. In contrast, a marked temperature-sensitive GC uptake by CHO cells expressing rat Oatp1 was observed (Fig. 4). Manipulations similar to those performed on HepG2 cells resulted in effects on pHi that were similar in wild-type and rat Oatp1-expressing cells. However, in some cases, these changes as well as those induced in GC uptake were very different to those observed in HepG2 cells (Fig. 5). Thus, the replacement of sodium by Chol induced an acidification of pHi (Fig. 5A) but this change was accompanied by a reduction in GC uptake (Fig. 5B). Similarly to what happened in HepG2 cells, amiloride also induced a significant decrease in pHi (Fig. 5A), but instead of enhancing GC uptake, this change in pHi was accompanied in Oatp1-expressing cells by a reduction in GC uptake (Fig. 5B). Partial replacement of sodium by either NH_4^+ or imidazole induced an acidification instead of an alkalinization of pHi (Fig. 5A). In agreement with the other manipulations that lowered pHi in these cells, this was also accompanied by an inhibition in GC uptake (Fig. 5B). The low amount of GC taken up by wild-type CHO cells was not significantly affected by any of the experimental circumstances described above (Fig. 5B). The study of GC uptake versus substrate concentrations also pointed to an inhibition when pHi was reduced from 7.45 to 7.30 while pHo remained at pH 7.40 (Fig. 6). The kinetic parameters obtained from fitting these results to a Michaelis–Menten equation revealed that those obtained in untreated cells were of the same order as those reported for this substrate by the authors who originally prepared these transfected cells [22]. In cells with relative intracellular acidification, a decrease in the value of the maximal velocity of transport (V_{max}), together with the absence of a significant change in the apparent affinity constant (K_m), were found (Table 2).

Table 2
Effect of pHi on kinetic parameters

pHi	7.45	7.30	*P*
V_{max} (pmol/60 s/mg protein)	294 ± 15	182 ± 5	< 0.05
K_m (μM)	81 ± 11	86 ± 14	n.s.
Intrinsic transport activity (V_{max}/K_m)	2.8	2.1	

Kinetic parameters (± S.D.) were determined from the plots shown in Fig. 6. GC uptake by CHO cells expressing rat Oatp1 was measured after 60 s incubation with GC concentrations ranging from 5 to 200 μM in the absence of amiloride (expected pHi approximately 7.45) or in the presence of 1 mM amiloride (expected pHi approximately 7.30).

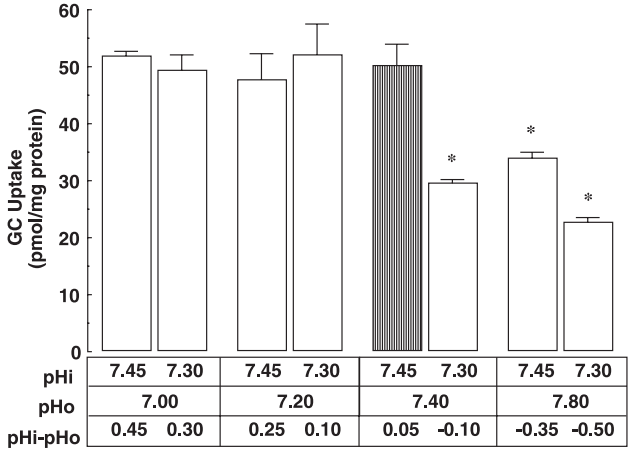

Fig. 7. Effect of extracellular (pHo) and intracellular pH (pHi) on [^{14}C]-glycocholic acid (GC) uptake by rat-Oatp1-expressing CHO cells. After removal of the culture medium, the cells were equilibrated at 37 °C for 60 min with NaCl-medium (96 mM NaCl, 5.3 mM KCl, 1.1 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.8 mM $CaCl_2$, 11 mM glucose, 50 mM Hepes, pH 7.40). Intracellular pH was not modified (pHi = 7.46 ± 0.03; i.e. approximately 7.45) or was reduced (pHi = 7.31 ± 0.02; i.e. approximately 7.30) by the addition of 1 mM amiloride 15 min immediately before uptake period. This began with the replacement of the incubation medium by another similar one in which pH had been adjusted to the indicated value. In all cases, the medium contained 10 μM GC (uptake measurements). Incubations were carried out for 90 s at 37 °C. To measure pHi, in separate plates, 1 mM [^{14}C]-dimethyloxazolidine dione plus [^{3}H]-H_2O (pHi experiments) were added at min 45 of the equilibration period. Each data point is the mean ± S.E. of 12 plates corresponding to four cultures in which measurements were performed on three plates per experimental condition and incubation time. *, $P < 0.05$, on comparing uptake values at pHo = 7.40 and at pHi = 7.45 by the paired *t*-test.

These changes, characteristic of non-competitive inhibition, led to a reduction in the efficiency of GC uptake or intrinsic transport activity, defined as the V_{max}/K_m.

To evaluate the effect of changes in the magnitude and direction of the proton gradient between the inside and outside of the cells, both pHi and pHo were altered. When pHi>pHo, regardless of the magnitude of the gradient, no effect on GC uptake was observed either at normal or decreased pHi (Fig. 7). When pHi < pHo, however, GC uptake was impaired, even when pHi was not lower than normal. No relationship between the reduction in GC uptake and the magnitude of the difference between pHi and pHo was observed (Fig. 7).

4. Discussion

When sodium-dependent mechanisms are involved in bile acid uptake, removal of sodium from the medium is expected to inhibit this process. However, a striking finding seen in the present study, that has also previously been observed by others [4,13], was the enhancement in GC uptake by HepG2 cells when sodium was replaced by Chol in the incubation medium. The amount of GC found in HepG2 cells at the end of the incubation period was the result of the net balance between uptake and efflux. Our results indicate that sodium substitution has no effect on GC efflux from previously loaded cells, which suggests that the accumulation of GC in these cells must be due to variations in the uptake mechanism. Therefore, in the ensuing discussion, we shall assume that manipulations to the composition of the incubation medium have no effect on GC export activity.

The fact that an enhanced GC uptake was also seen when different cations were used to replace sodium or even when a neutral sugar was used to adjust osmolarity after the removal of NaCl, indicates that stimulation was induced by the absence of sodium. Because all the circumstances found to stimulate GC uptake were accompanied by intracellular acidification, a relationship between both changes was suspected. Replacement of sodium by Chol or adding amiloride to the incubation medium of hepatoma cells have been shown to inhibit the Na^+/H^+ exchanger that normally prevents cellular acidification [32]. In rat hepatocytes, in which a situation of coexisting sodium-dependent and sodium-independent carriers hinders interpretation of the results, a pH-driven uptake of cholic acid has been reported [33]. Accordingly, a decreased pHi is expected to inhibit bile acid uptake by these cells. Indeed, previous studies have reported an inhibition of bile acid uptake by isolated rat hepatocytes [34] and perfused rat liver [35] when they were incubated with amiloride. Because sodium-dependent uptake is the predominant mechanism in these systems, the authors explained their results in terms of an amiloride-induced reduction in the transmembrane sodium gradient rather than as being due to the intracellular acidification. In any case, we observed the opposite phenomenon in HepG2 cells: that is, intracellular acidification resulted in enhanced GC uptake. This does not support a role for a pH-driven transport of GC in these cells.

Protonated forms of bile acids are expected to be more readily diffusible across the plasma membrane. This is in agreement with the findings of a pH gradient-driven cholate uptake by plasma membrane vesicles, which was interpreted as being due to non-ionic diffusion [36]. However, the p*Ka*′ value for GC is lower (≈ 4) than that of cholic acid (≈ 5) [37]. This means that at all pH values considered in the present study, GC was almost completely ionized. Hence, under these circumstances, it is unlikely that non-ionic diffusion may be involved in GC uptake by the cells.

If GC uptake were mediated by hydroxyl exchange, it could be speculated that a decrease in pHi would reduce the magnitude of the driving force required to energize GC transport and would therefore inhibit uptake. However, exactly the opposite occurred in HepG2 cells.

Thus, our results do not fully support any of the hypotheses discussed above. However, one explanation consistent with the results obtained in HepG2 cells is that there may be pHi-dependent mechanisms that directly or indirectly affect the function of sodium-independent bile acid carriers. One of these mechanisms could be the induction of changes in

the protonation state of the cytoplasmic domains of these transporters. This would explain why the repercussions in overall transport function may differ among the different carrier types that mediate sodium-independent GC uptake.

The results obtained in CHO 0-3 cells are also in agreement with the hypothesis of intracellular acidification-induced changes in sodium-independent bile acid transport, although some of the findings are also compatible with a reduction in the energy that would be necessary for GC uptake if this were mediated by a GC:OH^- exchange. Although organic anion transport by rat Oatp1 is believed to be sodium-independent, removal of this cation induced an inhibition in bile acid uptake by CHO 0-3 cells. Similar results have been reported by other authors who used either transiently expressed rat Oatp1 in HeLa cells [38] or *Xenopus laevis* oocytes injected with the cRNA of rat Oatp1 [20]. Moreover, bromosulfophthalein uptake by HeLa cells transfected with rat Oatp1 cDNA was sensitive to changes in the pH of the extracellular medium. This has lead to the suggestion that rat Oatp1 may function as an organic anion/OH^- exchanger or, alternatively, that this carrier contains sites that can be modified by H^+ [38]. In the present study, the intracellular acidification of CHO 0-3 cells was achieved, even by manoeuvres that caused alkalinization in HepG2 cells, probably due to the existence of differences in the mechanisms accounting for pHi homeostasis in both types of cells. In both cell lines, the values of pHi under control conditions are higher than in rat hepatocytes in primary culture [39]. This is probably related to the enhanced activity of the sodium/H^+ exchanger in proliferating cells [40,41]. Here, in all circumstances in which pHi was decreased, an inhibition in GC uptake was found. This is consistent with a reduction in the intracellular concentrations of OH^- able to energize an anion exchanger.

Moreover, kinetic studies revealed that the acidification of pHi elicited a reduction in V_{max}, with no marked effect on the apparent K_m. This is compatible with a lower translocation rate, due either to a reduced source of energy or to pHi-induced modifications in the carrier itself. If the first possibility were the predominant cause of the inhibition in GC uptake by CHO 0-3 cells, it could be expected that by increasing the magnitude of the gradient in the opposite direction, GC uptake would be augmented. However, when pHo was lowered below pHi, GC uptake was not affected, although when pHi decreased below pHo, GC uptake was decreased, but not in a manner proportional to the magnitude of the trans-membrane pH gradient.

In sum, our results indicate that intracellular acidification triggers changes in mechanisms accounting for sodium-independent bile acids transport. The possibilities of pHi-associated changes in carrier expression at midterm and that the modification of GC transport in response to intracellular acidification might be somehow cell-type specific cannot be ruled out. However, the most probable explanation for the direct effect of changes in pHi is the modification of the protonation state of intracellular domains of membrane proteins. This would result in either the stimulation or the inhibition of sodium-independent GC uptake, depending on the identity and nature of the carrier. The exact response of individual carriers OATP-A and OATP-8, as well as OATP-C and other members of this family of transporters to changes in pHi deserves to be investigated further, because these changes may have important implications in pathophysiological circumstances in which the control of pHi is impaired in liver cells and other tissues in which these carriers play a role in the transport of bile acids as well as of other endogenous and xenobiotic organic compounds.

Acknowledgements

The authors thank L. Muñoz de la Pascua, J.F. Martín Martín and J. Villoria Terrón for care of the animals. The secretarial work of M.I. Hernández and the technical assistance of E. Flores are gratefully acknowledged. Thanks are also due to N. Skinner for revision of the English text of the manuscript.

References

[1] P.J. Meier, B. Stieger, Bile salt transporters, Annu. Rev. Physiol. 64 (2002) 635–661.
[2] B. Hagenbuch, P.J. Meier, The superfamily of organic anion transporting polypeptides, Biochim. Biophys. Acta 1609 (2003) 1–18.
[3] J.L. Boyer, B. Hagenbuch, M. Ananthanarayanan, F. Suchy, B. Stieger, P.J. Meier, Phylogenic and ontogenic expression of hepatocellular bile acid transport, Proc. Natl. Acad. Sci. U. S. A. 90 (1993) 435–438.
[4] G.A. Kullak-Ublick, U. Beuers, G. Paumgartner, Molecular and functional characterization of bile acid transport in human hepatoblastoma HepG2 cells, Hepatology 23 (1996) 1053–1060.
[5] G.A. Kullak-Ublick, J. Glasa, C. Boker, M. Oswald, U. Grutzner, B. Hagenbuch, B. Stieger, P.J. Meier, U. Beuers, W. Kramer, G. Wess, G. Paumgartner, Chlorambucil taurocholate is transported by bile acid carriers expressed in human hepatocellular carcinomas, Gastroenterology 113 (1997) 1295–1305.
[6] O. Briz, M.A. Serrano, N. Rebollo, B. Hagenbuch, P.J. Meier, H. Koepsell, J.J.G. Marin, Carriers involved in targeting the cytostatic bile acid-cisplatin derivatives *cis*-diammine-chloro-cholylglycinate-platinum(II) and *cis*-diammine-bisursodeoxycholate-platinum(II) toward liver cells, Mol. Pharmacol. 61 (2002) 853–860.
[7] G. Fricker, G. Hugentobler, P.J. Meier, G. Kurz, J.L. Boyer, Identification of a single sinusoidal bile salt uptake system in skate liver, Am. J. Physiol. 253 (1987) G816–G822.
[8] C.M.I. Rabergh, K. Ziegler, B. Isomaa, M.M. Lipsky, J.E. Eriksson, Uptake of taurocholic acid and cholic acid in isolated hepatocytes from rainbow trout, Am. J. Physiol. 267 (1994) G380–G386.
[9] F.J. Suchy, J.C. Bucuvalas, A.L. Goodrich, M.S. Moyer, B.L. Blitzer, Taurocholate transport and Na–K-ATPase activity in fetal and neonatal rat liver plasma membrane vesicles, Am. J. Physiol. 251 (1986) G665–G673.
[10] C. Dubuisson, D. Cresteil, M. Desrochers, D. Decimo, M. Hadchouel, E. Jacquemin, Ontogenic expression of the Na^+ independent organic anion transporting polypeptide (Oatp) in rat liver and kidney, J. Hepatol. 25 (1996) 932–940.
[11] R.C. De Belle, V. Vaupshas, B.B. Vitullo, L.R. Haber, E. Shaffer, G.G. Mackie, H. Owen, J.M. Little, R. Lester, Intestinal absorption of bile salts: immature development in the neonate, J. Pediatr. 94 (1979) 472–476.

[12] B.L. Schneider, P.A. Dawson, D.M. Christie, W. Hardikar, M.H. Wong, F.J. Suchy, Cloning and molecular characterization of the ontogeny of a rat ileal sodium-dependent bile acid transporter, J. Clin. Invest. 95 (1995) 745–754.
[13] T.K. Lee, C.L. Hammond, N. Ballatori, Intracellular glutathione regulates taurocholate transport in HepG2 cells, Toxicol. Appl. Pharmacol. 174 (2001) 207–215.
[14] L.M. Satlin, V. Amin, A.W. Wolkoff, Organic anion transporting polypeptide mediates organic anion/HCO_3^- exchange, J. Biol. Chem. 272 (1997) 26340–26345.
[15] B.L. Blitzer, C. Terzakis, K.A. Scott, Hydroxyl/bile acid exchange. A new mechanism for the uphill transport of cholate by basolateral liver plasma membrane vesicles, J. Biol. Chem. 261 (1986) 12042–12046.
[16] L. Diewald, J. Rupp, M. Dreger, F. Hucho, C. Gillen, H. Nawrath, Activation by acidic pH of CLC-7 expressed in oocytes from *Xenopus laevis*, Biochem. Biophys. Res. Commun. 291 (2002) 421–424.
[17] D.P. Aden, A. Fogel, S. Plotkin, I. Damjanov, B.B. Knowles, Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line, Nature 282 (1979) 615–616.
[18] G.T. Everson, M.A. Polokoff, A human hepatoblastoma cell line exhibiting defects in bile acid synthesis and conjugation, J. Biol. Chem. 261 (1986) 2197–2201.
[19] S. Goenner, A. Boutron, T. Soni, A. Lemonnier, N. Moatti, Amino acid transport systems in the human hepatoma cell line HepG2, Biochem. Biophys. Res. Commun. 189 (1992) 472–479.
[20] E. Jacquemin, B. Hagenbuch, B. Stieger, A.W. Wolkoff, P.J. Meier, Expression cloning of a rat liver Na^+-independent organic anion transporter, Proc. Natl. Acad. Sci. U. S. A. 91 (1994) 133–137.
[21] G.A. Kullak-Ublick, B. Hagenbuch, B. Stieger, A.W. Wolkoff, P.J. Meier, Functional characterization of the basolateral rat liver organic anion transporting polypeptide, Hepatology 20 (1994) 411–416.
[22] U. Eckhardt, A. Schroeder, B. Stieger, M. Hochli, L. Landmann, R. Tynes, P.J. Meier, B. Hagenbuch, Polyspecificity substrate uptake by the hepatic organic anion transporter oatp1 in stably transfected CHO cells, Am. J. Physiol. 276 (1999) G1037–G1042.
[23] D.P. Palermo, M.E. De Graaf, D.R. Marotti, E. Rehberg, L.E. Post, Production of analytical quantities of recombinant proteins in Chinese hamster ovary cells using sodium butyrate to elevate gene expression, J. Biotechnol. 19 (1991) 35–48.
[24] B. Vasquez, F. Ishibashi, B.V. Howard, Measurement of intracellular volume in monolayers of cultured cells, In Vitro 18 (1982) 643–649.
[25] R.A. Iles, R.D. Cohen, The effect of varying the amount of unlabelled 5,5-dimethyloxazocidine-2,4-dione (DMO) in the measurement of rat hepatic intracellular pH using [^{14}C]-DMO, Clin. Sci. Mol. Med. 46 (1974) 277–280.
[26] J.J.G. Marin, M. Corbic, M. Dumont, G. De Couet, S. Erlinger, Role of H^+ transport in ursodeoxycholate-induced biliary HCO_3^- secretion in the rat, Am. J. Physiol. 249 (1985) G335–G341.
[27] M.A.K. Markwell, S.M. Haas, L.L. Bieber, N.E. Tolbert, A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples, Anal. Biochem. 87 (1978) 206–210.
[28] J. König, Y. Cui, A.T. Nies, D. Keppler, Localization and genomic organization of a new hepatocellular organic anion transporting polypeptide, J. Biol. Chem. 275 (2000) 23161–23168.
[29] J.L. Kinsella, P.S. Aronson, Amiloride inhibition of the Na^+–H^+ exchanger in renal microvillus membrane vesicles, Am. J. Physiol. 241 (1981) F374–F379.
[30] H. Mizuuchi, T. Katsura, K. Ashida, Y. Hashimoto, K.I. Inui, Diphenhydramine transport by pH-dependent tertiary amine transport system in Caco-2 cells, Am. J. Physiol. 278 (2000) G563–G569.
[31] J.S. Smith, C.S. Pace, Modification of glucose-induced insulin release by alteration of pH, Diabetes 23 (1983) 61–66.
[32] W.H. Weintraub, T.E. Machen, pH regulation in hepatoma cells—roles for Na^+–H^+ exchange, Cl^-–HCO_3^- exchange, and Na^+–HCO_3^- cotransport, Am. J. Physiol. 257 (1989) G317–G327.
[33] C.M. Veith, T. Thalhammer, F.X. Felberbauer, J. Graf, Relationship of hepatic cholate transport to regulation of intracellular pH and potassium, Biochim. Biophys. Acta 1103 (1992) 51–61.
[34] M.S. Anwer, A.U. Branson, J.M. Atkinson, Mechanism of inhibition of hepatic bile acid uptake by amiloride and 4,4′-diisothiocyano-2,2′-disulfonic stilbene (DIDS), Biochem. Pharmacol. 42 (1991) S135–S141.
[35] M.S. Anwer, J.M. Atkinson, P. Zimniak, Amiloride and taurine inhibit cholate-induced HCO_3^--rich choleresis in perfused rat livers, Am. J. Physiol. 259 (1990) G453–G461.
[36] C. Caflisch, B. Zimmerli, J. Reichen, P.J. Meier, Cholate uptake in basolateral rat liver plasma membrane vesicles and in liposomes, Biochim. Biophys. Acta 1021 (1990) 70–76.
[37] D.M. Small, The physical chemistry of cholanoic acids, in: P.P. Nair, D. Kritchevsky (Eds.), The Bile Acids. Chemistry, Physiology and Metabolism, vol. 1, Plenum Press, New York, 1971, pp. 249–256.
[38] N. Kanai, R. Lu, Y. Bao, A.W. Wolkoff, V.L. Schuster, Transient expression of Oatp organic anion transporter in mammalian cells: identification of candidate substrates, Am. J. Physiol. 39 (1996) F319–F325.
[39] A.S. Pollock, Intracellular pH of hepatocytes in primary monolayer culture, Am. J. Physiol. 15 (1984) F738–F744.
[40] S.J. Reshkin, A. Bellizzi, S. Caldeira, V. Albarani, I. Malanchi, M. Poignee, M. Alunni-Fabbroni, V. Casavola, M. Tommasino, Na^+/H^+ exchanger-dependent intracellular alkalinization is an early event in malignant transformation and plays an essential role in the development of subsequent transformation-associated phenotypes, FASEB J. 14 (2000) 2185–2197.
[41] D. Rotin, D. Steele-Norwood, S. Grinstein, I. Tannock, Requirement of the Na^+/H^+ exchanger for tumor growth, Cancer Res. 49 (1989) 205–211.