

# Further Characterization of the Electrogenicity and pH Sensitivity of the Human Organic Anion-Transporting Polypeptides OATP1B1 and OATP1B3

Pablo Martinez-Becerra, Oscar Briz, Marta R. Romero, Rocio I. R. Macias, Maria J. Perez, Carlos Sancho-Mateo, Maria P. Lostao, Jose M. Fernandez-Abalos, and Jose J. G. Marin

Laboratory of Experimental Hepatology and Drug Targeting (P.M.-B., O.B., M.R.R., R.I.R.M., M.J.P., J.J.G.M.) and Department of Microbiology and Genetics (J.M.F.-A.), University of Salamanca, and Research Unit, University Hospital of Salamanca (O.B., M.J.P.), Salamanca, Spain; Department of Nutrition, Food Science, Physiology and Toxicology, University of Navarra, Pamplona, Spain (C.S.-M., M.P.L.); Instituto de Estudios de Salud de Castilla y Leon, Salamanca, Spain (M.J.P.); and Centro de Investigación Biomédica en Red en el Área temática de Enfermedades Hepáticas y Digestivas, Instituto de Salud Carlos III, Madrid, Spain (P.M.B., O.B., M.R.R., R.I.R.M., M.J.P., J.J.G.M.)

Received November 12, 2010; accepted December 20, 2010

## ABSTRACT

Organic anion-transporting polypeptides (OATPs) are involved in the liver uptake of many endogenous and xenobiotic compounds, such as bile acids and drugs, respectively. Using *Xenopus laevis* oocytes and Chinese hamster ovary (CHO) cells expressing rat Oatp1a1, human OATP1B1, or OATP1B3, the sensitivity of these transporters to extracellular/intracellular pH (pHo/pHi) and changes in plasma membrane potential ( $\Delta\psi$ ) was investigated. In *X. laevis* oocytes, nonspecific plasma membrane permeability increased only at pHo below 4.5. Above this value, both using oocytes and CHO cells, extracellular acidification affected differently the specific transport of taurocholic acid (TCA) and estradiol 17 $\beta$ -D-glucuronide (E<sub>2</sub>17 $\beta$ G) by Oatp1a1 (stimulation), OATP1B1 (inhibition), and OATP1B3 (stimulation). Changes in substrate uptake in the presence of valinomycin (K<sup>+</sup>-ionophore), carbonyl cyanide 3-chlorophenylhydrazone and nigericin (protonophores), and amiloride (Na<sup>+</sup>/H<sup>+</sup>-inhibitor) and cation replacement in the

medium were studied with fluorescent probes for measuring substrate uptake (cholyglycyl amidofluorescein) and changes in pH<sub>i</sub> (SNARF-4F) and  $\Delta\psi$  [DiIC<sub>4</sub>(5)]. The results suggest that activity of these three carriers is sodium/potassium-independent and affected differently by changes in pHo and  $\Delta\psi$ : Oatp1a1 was confirmed to be an electroneutral anion exchanger, whereas the function of both OATP1B1 and OATP1B3 was markedly affected by the magnitude of  $\Delta\psi$ . Moreover, electrophysiological measurements revealed the existence of a net anion influx associated to OATP1B1/OATP1B3-mediated transport of TCA, E<sub>2</sub>17 $\beta$ G, and estrone-3-sulfate. Furthermore, a leakage of Na<sup>+</sup> through OATP1B1 and OATP1B3, which is not coupled to substrate transport, was found. In conclusion, these results suggest that OATP1B1 and OATP1B3 are electrogenic transporters whose activity may be strongly affected under circumstances of displacement of local pH.

This study was supported in part by the Instituto de Salud Carlos III, Fondo de Investigaciones Sanitarias [Grants CP05/0135, PI070517, and PI080151]; the Junta de Castilla y Leon [Grant GR75-2008]; Ministerio de Ciencia e Innovación, Plan Nacional de Investigación Científica, Desarrollo e Innovación Tecnológica [Grants BFU2006-12577, SAF2009-08493, SAF2010-15517]; Fundación Investigación Médica Mutua Madrileña [Conv-VI, 2009]; Fundación Marcelino Botín. P.M.-B. is the recipient of a predoctoral fellowship from the Ministerio de Ciencia e Innovación (Grant AP2006-02465) and C.-S.M. is from "Asociación de Amigos" University of Navarra, Spain. The group is member of the Network for Cooperative Research on Membrane Transport Proteins (REIT), cofunded by the Ministerio de Ciencia e Innovación, Spain, and the European Regional Development Fund [Grant BFU2007-30688-E/BFI] and belongs to the CIBERehd (Centro de Investigación Biomédica en Red) for Hepatology and Gastroenterology Research (Instituto de Salud Carlos III).

Article, publication date, and citation information can be found at <http://molpharm.aspetjournals.org>.  
doi:10.1124/mol.110.069971.

## Introduction

The liver is involved in the detoxification of a large variety of endogenous and xenobiotic compounds (Meier and Stieger, 2002; Kullak-Ublick et al., 2004). In addition to Na<sup>+</sup>-taurocholate cotransporting polypeptide (NTCP, gene symbol *SLC10A1*) (Hagenbuch et al., 1991), several sodium-independent transporters account for the liver uptake of bile acids and other cholephilic compounds. These belong to the *SLCO* and *SLC22A* gene families, whose products are, respectively, organic anion-transporting polypeptides (OATPs) (Hagenbuch and Meier, 2003) and two groups of transporters classified as organic anion-transporters (Suzuki and Sugiyama,

**ABBREVIATIONS:** NTCP, Na<sup>+</sup>-taurocholate cotransporting polypeptide; OATP, organic anion-transporting polypeptide;  $\Delta\psi$ , plasma membrane potential; pHo, extracellular pH; TCA, taurocholic acid; E3S, estrone-3-sulfate; E<sub>2</sub>17 $\beta$ G, estradiol 17 $\beta$ -D-glucuronide; CCCP, carbonyl cyanide 3-chlorophenylhydrazone; CGamF, cholyglycyl amidofluorescein; MES, 2-(N-morpholino)ethanesulfonic acid; pH<sub>i</sub>, intracellular pH.

2000) and organic cation transporters (Koepsell and Endou, 2004).

Despite the interest in these transporters in physiology and pharmacology, important characteristics, such as their driving force and the transport mechanism, are not well known for most of them (Ballatori et al., 2005). On the basis of comparative analyses of typical OATPs, some authors have proposed a representative structural model for these transporters, which essentially consists of a central and positively charged pore through which the substrate is translocated (Meier-Abt et al., 2005). Regarding sensitivity to plasma membrane potential ( $\Delta\Psi$ ) and electrogenicity, Oatp/OATPs are generally believed to behave as electro-neutral anion exchangers. However, clear support for this concept has been obtained only for rat Oatp1a1. This has been reported to work by coupling the uptake of organic anions to the efflux of bicarbonate (Satlin et al., 1997; Leuthold et al., 2009) or glutathione (Li et al., 1998) and is hence sensitive to stimulation by extracellular protons (Kanai et al., 1996; Leuthold et al., 2009). The efflux of intracellular glutathione or glutathione *S*-conjugates has been suggested to activate Oatp1a4-mediated uptake (Li et al., 2000). Moreover, transport mediated by either Oatp1a1 or Oatp1a4 is potentially bidirectional (Shi et al., 1995; Li et al., 2000), which implies that the overall directionality of the transport would depend on the gradient of the predominant substrate.

Studies in human hepatoblastoma HepG2 cells that express several OATP isoforms have indicated that intracellular glutathione modulates both the uptake and efflux of cholephilic organic anions (Lee et al., 2001). Moreover, a pH-driven transport of bile acids in these cells has also been reported (Marin et al., 2003). Although OATP1B3 has recently been suggested to be sensitive to activation by glutathione (Briz et al., 2006), this could not be confirmed by other authors (Mahagita et al., 2007). The reason for these controversial results may be due in part to the existence of artifactually induced acidification of local pH by addition of glutathione to insufficiently buffered media (Briz et al., 2006). This is consistent with the recently described effect of extracellular pH (pHo) on the function of several OATP/Oatps (Leuthold et al., 2009).

OATP2B1 exhibits a dual response to changes in pHo. OATP2B1-mediated taurocholic acid (TCA) uptake is activated at acidic pH (Nozawa et al., 2004), whereas at physiological pH, OATP2B1 is unable to recognize bile acids as substrates (Kullak-Ublick et al., 2001). In human embryonic kidney 293 cells expressing OATP2B1 the transport of estrone-3-sulfate (E3S) mediated by this carrier is enhanced by an inwardly directed proton gradient (Sai and Tsuji, 2004) and inhibited by the proton ionophore FCCP (Nozawa et al., 2004).

The sensitivity of OATPs to pHo may play a role in drug uptake, because in some territories, the pHo may differ from the common values, which may determine the amount of drug reaching pharmacologically active levels in targeted cells. For instance, a common feature of inflammatory foci is local acidosis as a result of the increased lactic acid production by infiltrated neutrophils and/or to the presence of fatty acid products from bacterial metabolism (Grinstein et al., 1991). Moreover, rapid tumor cell growth together with hypoxic conditions determine an active glycolytic metabolism

that also results in acidification to pH values below 6.0 in the interstitial fluid (Kraus and Wolf, 1996). Accordingly, drug uptake through OATP1B1 and/or OATP1B3 in tumors expressing these transporters (for review, see Marin et al., 2009) could be affected by pHo. Although under physiological circumstances the expected magnitude of changes in pHo in liver parenchyma is small, acid-base disorders occurring in the setting of liver disease (Ahya et al., 2006), renal dysfunction, and acute intoxication with salicylates, methanol, or ethylene glycol (Forsythe and Schmidt, 2000) may decrease pHo and hence affect the transport ability of OATP1B1 and OATP1B3.

The aim of the present study was to further characterize the dependence on  $\Delta\Psi$  and pHo of the transport mechanism of two important OATP isoforms in human liver, OATP1B1 and OATP1B3.

## Materials and Methods

**Chemicals.** [ $^3\text{H}$ ]Estradiol 17 $\beta$ -D-glucuronide ( $\text{E}_2$ 17 $\beta$ G; specific activity, 45 Ci/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). [ $^3\text{H}$ ]Inulin (specific activity, 304.8 Ci/g), [ $^3\text{H}$ ]TCA (specific activity, 4.6 Ci/mmol), [ $^3\text{H}$ ]E3S (specific activity, 54 Ci/mmol), and [ $^{14}\text{C}$ ]mannitol (specific activity, 55 Ci/mol) were obtained from PerkinElmer Life and Analytical Sciences (Waltham, MA). Nigericin and the fluorescent probes 5-(and-6)-carboxylic acid acetoxymethyl ester (SNARF-4F) and the carbocyanine DiI $\text{C}_1(5)$  were obtained from Invitrogen (Carlsbad, CA). Amiloride, carbonyl cyanide 3-chlorophenylhydrazone (CCCP), G418 (Geneticin), sodium butyrate, Triton X-100, valinomycin, and unlabeled  $\text{E}_2$ 17 $\beta$ G, E3S, and TCA were purchased from Sigma-Aldrich (St. Louis, MO). According to the suppliers, the purity of these compounds was  $\geq 98\%$ . The fluorescent bile acid derivative cholyglycyl amidofluorescein (CGamF) was synthesized by coupling the amido group of fluorescein isothiocyanate to the carboxyl group of the glycine moiety of glycocholic acid (Sherman and Fisher, 1986). Using semipreparative thin layer chromatography, CGamF was purified twice. This reduced contamination by fluorescein isothiocyanate and glycocholic acid below detectable levels by analytical thin layer chromatography. All other reagents were of analytical grade.

**Protein Detection By Immunofluorescence Assays.** Immunofluorescence studies were carried out using wild-type or OATP/Oatp-expressing oocytes 48 h after microinjection of cRNA. Groups of three oocytes were fixed as described previously by Nakanishi et al. (2003). Sections of 5  $\mu\text{m}$  were permeabilized in ice-cold methanol for 1 min, and nonspecific binding sites were blocked by incubation with 5% fetal calf serum for 30 min. Preparations were incubated at room temperature for 1 h with the following primary antibodies: monoclonal antibodies against OATP1B1 (clone ESL), purchased from Abcam (Cambridge, UK); OATP1B3 (clone mMDQ), from PROGEN Biotechnik (Heidelberg, Germany); and Oatp1a1 (K-10) kindly provided by Drs. P. Meier, B. Stieger, and B. Hagenbuch (Department of Clinical Pharmacology, Zurich University Hospital, Zurich, Switzerland). Fluorescence staining with the appropriate anti-mouse or rabbit Ig Alexa 488-conjugated secondary antibodies (Invitrogen) was visualized using a laser-scanning confocal microscope (TCS SP2; Leica, Wetzlar, Germany).

**Synthesis of cRNAs for Injection into Oocytes.** Recombinant plasmids containing the open reading frame of rat Oatp1a1 and human NTCP, both cloned in pSPORT1, and of human OATP1B1 and OATP1B3, both cloned in pCMV6-XL4, were kindly donated by Drs. Meier, Stieger, and Hagenbuch. After amplification in *Escherichia coli*, these plasmids were isolated using the Qiagen Plasmid Mini kit (Izasa) and further linearized with appropriate restriction enzymes. Capped and poly(A)-tailed cRNAs were synthesized using the T7 mMessage mMachine Ultra kit (Ambion, Austin, TX).

**Preparation of *X. laevis* Oocytes.** Mature female frogs (*X. laevis*) purchased from Regine Olig (Hamburg, Germany) and Xenopus Express (Le Bourg, France) were used. The animals received humane care as outlined in the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996), and were treated in accordance with current Spanish (RD 223/1988) and European Union (86/609/CEE) laws. Experimental protocols were approved and supervised by the Ethical Committee for Laboratory Animals of the University of Salamanca and University of Navarra.

The harvesting and preparation of oocytes were carried out as described elsewhere (Briz et al., 2002). Defolliculated stage V and VI oocytes were injected with 9 ng of cRNA dissolved in Tris-EDTA buffer (1 mM EDTA and 10 mM Tris, pH 8.0), and were subsequently cultured at 18°C in modified Barth's solution [88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO<sub>3</sub>, 0.82 mM MgSO<sub>4</sub>, 0.33 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 0.41 mM CaCl<sub>2</sub>, and 15 mM HEPES, pH 7.6], supplemented with gentamicin (50 mg/l), penicillin (100 U/ml), streptomycin (100 mg/l), and amphotericin B (0.25 mg/l) at 18°C for 2 days. This time was selected on the basis of the results of preliminary experiments addressing the time course of the functional expression of these carriers (data not shown).

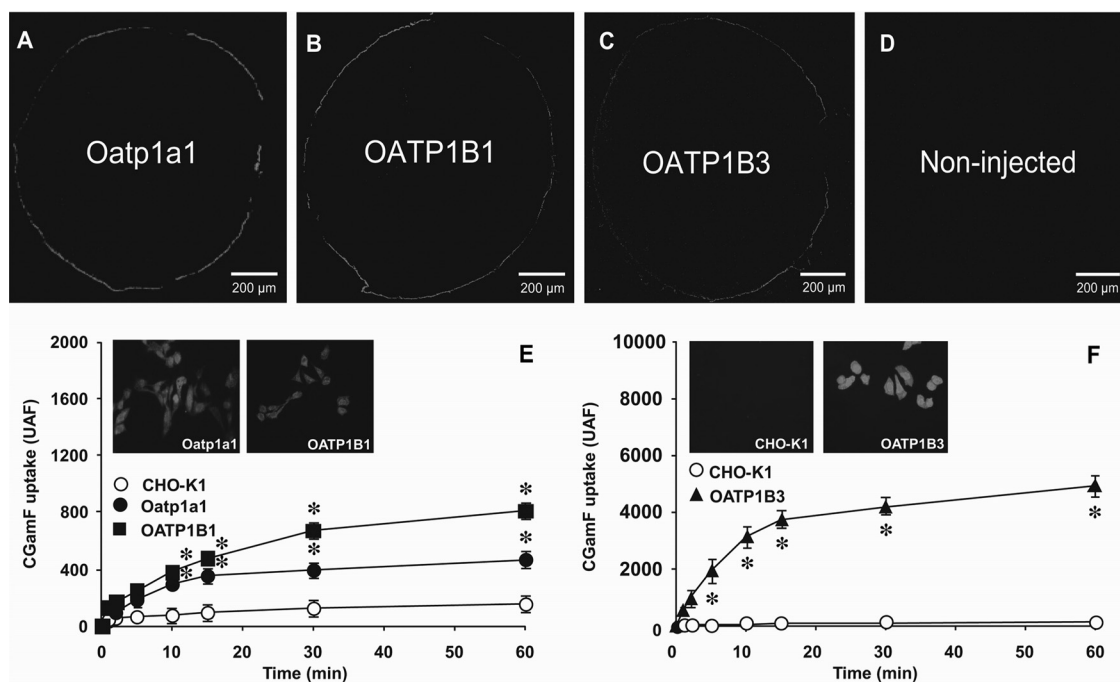
Oocytes were washed with substrate-free uptake medium containing HEPES or MES as buffer (100 mM choline chloride, 2 mM KCl, 1 mM CaCl<sub>2</sub>, 1 mM MgCl<sub>2</sub>, and 10 mM HEPES/10 mM MES, pH 7.5) and incubated with 100 µl of uptake medium containing the desired amount of the substrate to be tested at 25°C for the indicated time. Uptake was stopped by the addition of 4 ml of ice-cold uptake medium. The oocytes were washed three more times with 4 ml of ice-cold uptake medium before being placed individually in vials and dissolved in 200 µl of 10% SDS for at least 2 h to subsequently measure the amount of radioactive substrate by liquid scintillation on a liquid scintillation counter (LS-6500; Beckman Coulter, Fullerton, CA). Noninjected oocytes were used to measure nonspecific uptake, because in previous studies, no significant difference with

water-injected oocytes was observed (Briz et al., 2006). In some experiments, net uptake was calculated from the difference in uptake values between noninjected oocytes and those expressing the desired transporter.

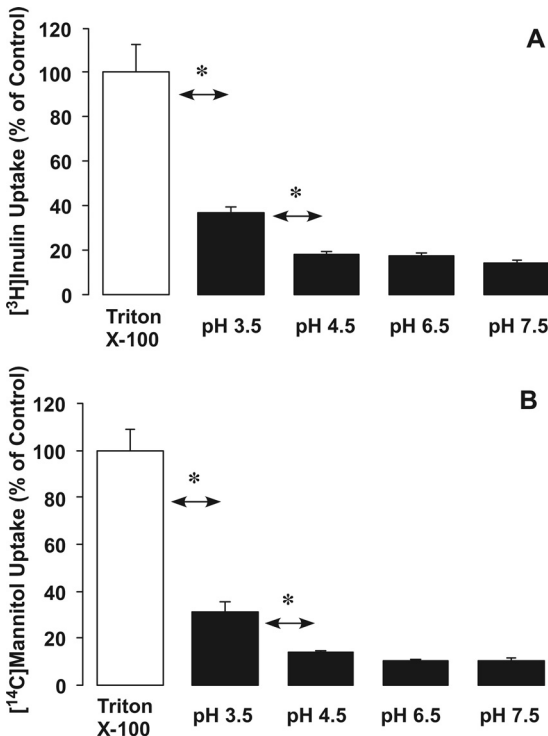
**Effect of Extracellular pH and Manipulation of the Medium on TCA and E<sub>2</sub>17βG Transport in *X. laevis* Oocytes.** To investigate the effect of the pH<sub>o</sub> on the transport activity of Oatp1a1, OATP1B1, and OATP1B3, the pH of uptake medium was adjusted to 6.5, 4.5, or 3.5 using HCl. The low buffering ability of buffered HEPES solution at pH 5.5 precluded use of this value with consistent results. To adjust the pH of the solution to pH 5.5, we used MES buffer, which is more suitable than HEPES to maintain pH within the 5 to 7 range. The effect of the pH<sub>o</sub> on nonspecific permeabilization of oocyte plasma membranes was investigated by measuring [<sup>3</sup>H]inulin and [<sup>14</sup>C]mannitol uptake by oocytes incubated with uptake medium at several pH values with either of these compounds at 25°C for 1 h. Similar experiments in the presence of 0.05% Triton X-100 were performed as positive controls of membrane permeabilization and [<sup>3</sup>H]inulin and [<sup>14</sup>C]mannitol uptake under these circumstances, considered arbitrarily as 100%.

To determine ion, proton, and voltage dependence, choline was replaced by sodium or potassium. Moreover, the K<sup>+</sup>- and H<sup>+</sup>-ionophores valinomycin (10 µM), nigericin (10 µM), and CCCP (50 µM) were added to the uptake medium.

**Electrophysiology Experiments.** Mature *X. laevis* oocytes were injected with cRNA coding for NTCP (used here as a positive control), OATP1B1, OATP1B3, or rat Oatp1a1 dissolved in Tris-EDTA buffer and were subsequently cultured in modified Barth's solution at 18°C for 2 days. Noninjected oocytes served as controls. The electrophysiology measurements were performed at room temperature using an adaptation of the two-electrode voltage-clamp method (Larrayoz et al., 2006). Oocytes were perfused with uptake medium at pH 7.5 containing 100 mM NaCl (Na<sup>+</sup> buffer) instead of choline chloride (choline buffer). In some experiments, the perfused buffer contained



**Fig. 1.** Illustration of the expression of OATP/Oatp transporters in *X. laevis* oocytes and CGamF uptake by CHO cells. Confocal fluorescence microscopy of *X. laevis* oocytes expressing Oatp1a1 (A), OATP1B1 (B), or OATP1B3 (C). *X. laevis* oocytes were injected with the corresponding cRNA of interest 2 days before fluorescence visualization. Noninjected oocytes were used as negative controls (D). Time course of CGamF uptake by wild-type CHO cells (E and F) or cells expressing Oatp1a1 (E), OATP1B1 (E), or OATP1B3 (F). The cells were incubated in uptake medium containing 1 µM CGamF (for OATP1B3) or 5 µM (for Oatp1a1 and OATP1B1) at 37°C for 15 min. Uptake values are means ± S.D. from at least nine determinations per data point, obtained using cells from three different cultures. \*,  $p < 0.05$ , on comparing OATP-expressing CHO cells with wild-type cells by the paired  $t$  test. The insets depict a fluorescence microscopy picture representative of each group after incubating the cells with 10 µM CGamF at 37°C for 15 min.

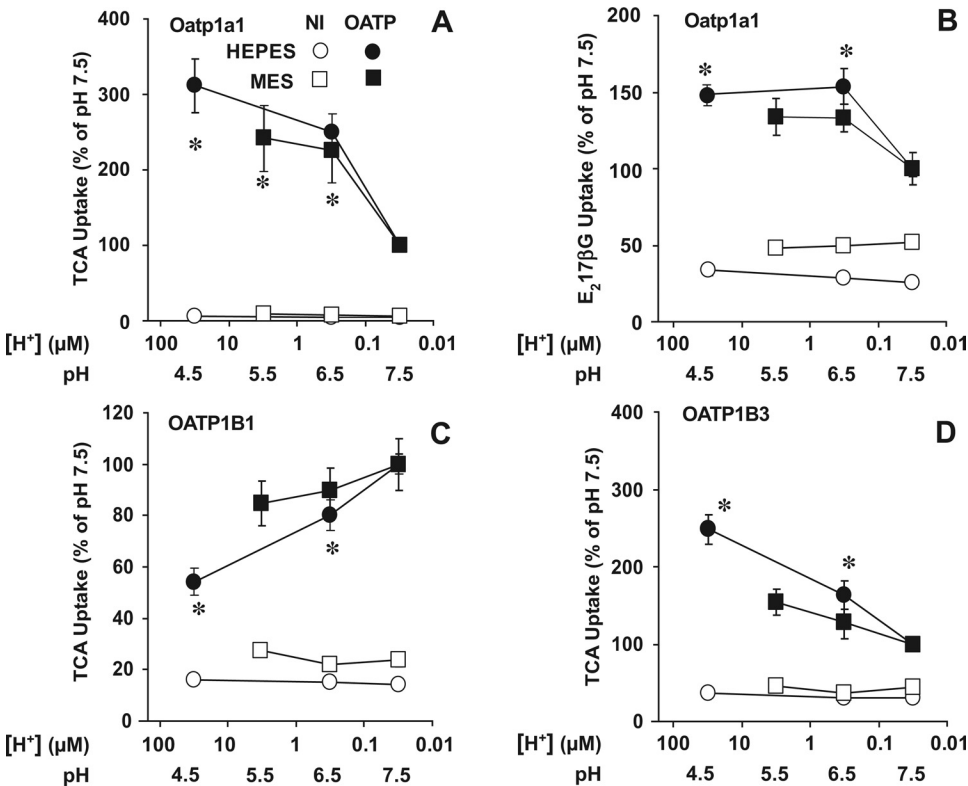


**Fig. 2.** Effect of pH on oocyte plasma membrane permeability. Noninjected oocytes were incubated with uptake medium containing 120  $\mu$ M [<sup>3</sup>H]inulin (A) or 30  $\mu$ M [<sup>14</sup>C]mannitol (B) at 25°C for 1 h. Results are expressed as percentages of the amount of radiolabeled substrate accumulated in oocytes permeabilized by incubation with 0.05% Triton X-100 in uptake medium, pH 7.5, at 25°C for 1 h. Values are means  $\pm$  S.D. from at least 24 determinations per data point obtained using oocytes from three different frogs. \*,  $p < 0.05$ , by the Bonferroni method of multiple range testing.

100 mM choline + Na<sup>+</sup>, which was obtained by combining both cations in different proportions (25, 50, and 75%). The oocyte membrane potential was held at -50 mV, and continuous current data were recorded using an Axoscope v1.1.1.14 device (Molecular Devices, Sunnyvale, CA).

**Effect of pH on TCA Uptake by CHO Cells.** Chinese hamster ovary (CHO) cells, either wild-type (CHO-K1) or stably transfected (with the cDNA of rat Oatp1a1, human OATP1B1 or OATP1B3), were generously donated by Drs. Meier, Stieger, and Hagenbuch (Gui et al., 2008). The cells were seeded ( $4 \times 10^5$  cells per 3.5-cm diameter dish) and cultured in Dulbecco's modified Eagle's medium (10 g/l) supplemented with 10% fetal calf serum, 10 mM NaHCO<sub>3</sub>, 2 mM L-glutamine, 0.43 mM L-proline, and antibiotic cocktail and used after 3 days in culture at subconfluence. The culture medium for stably transfected CHO cells also contained 400  $\mu$ g/ml G-418. Transporter expression was stimulated by adding 5 mM sodium butyrate to the culture medium 24 h before carrying out the experiments (Palermo et al., 1991). Before using these cells in transport studies, they were incubated with an equilibration medium (96 mM NaCl, 5.3 mM KCl, 1.1 mM KH<sub>2</sub>PO<sub>4</sub>, 0.8 mM MgSO<sub>4</sub>, 1.8 mM CaCl<sub>2</sub>, 11 mM glucose, and 50 mM HEPES, pH 7.4) at 37°C for 30 min. The medium was replaced by a fresh one, at the desired pH (6.5 or 7.4), containing 10  $\mu$ M [<sup>3</sup>H]TCA at 37°C, which was removed 15 min later. The cells were washed four times with 1 ml of prechilled radioactivity-free equilibration medium. Finally, 1 ml of Lowry medium (100 mM NaOH and 189 mM Na<sub>2</sub>CO<sub>3</sub>) was added and the cells were scraped off the plates to measure the amount of radioactive substrate. Proteins were determined by a modification of the Lowry method, using bovine serum albumin as a standard (Markwell et al., 1978). Values of extracellular pH lower than 6.5 were not used in further experiments in view of the alterations observed by flow cytometry in cell size (forward-scattered light) and internal complexity (side-scattered light) when the cells were incubated at pH 5.5 (data not shown).

**Flow Cytometry Experiments.** Flow cytometry on a FACSCalibur flow cytometer (BD Biosciences, Madrid, Spain) was carried out to determine cellular uptake of fluorescent compounds. CGamF was used as the substrate of Oatp1a1, OATP1B1, and OATP1B3. SNARF-4F and

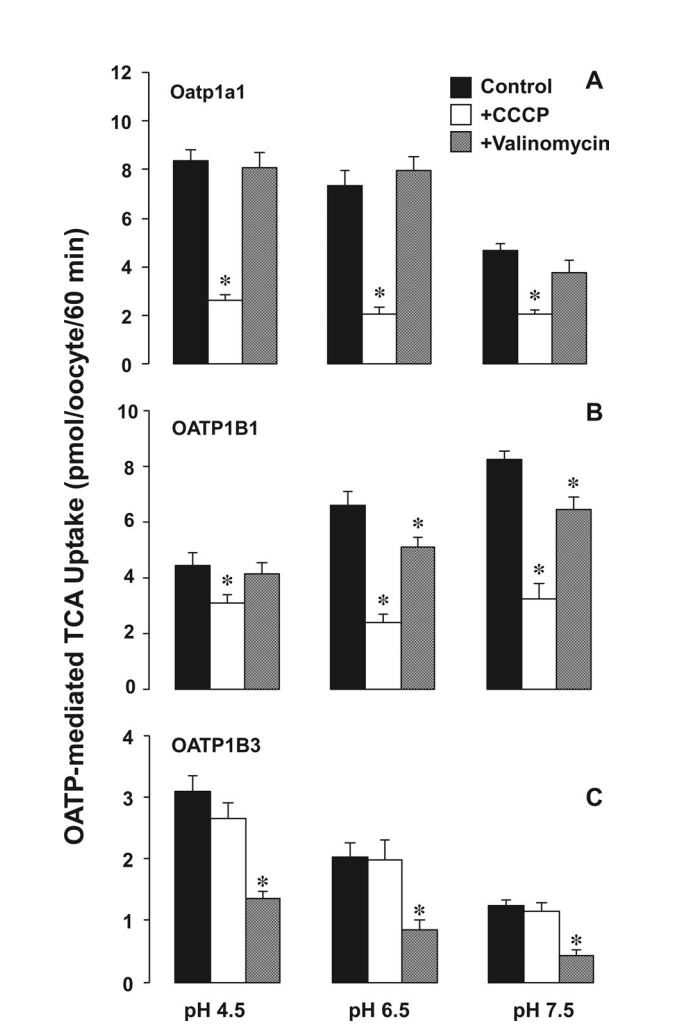


**Fig. 3.** Effect of pH on Oatp1a1, OATP1B1-, and OATP1B3-mediated transport. Noninjected *X. laevis* oocytes (NI), or oocytes expressing Oatp1a1 (A and B), OATP1B1 (C), or OATP1B3 (D) were incubated in uptake medium containing 10 mM HEPES or MES and 10  $\mu$ M concentrations of the desired radiolabeled substrate TCA or E<sub>2</sub> 17βG at 25°C for 1 h. Results are expressed as percentages of the amount of substrate taken up by the oocytes incubated in uptake medium containing HEPES or MES at pH 7.5. Values are means  $\pm$  S.D. from at least 24 determinations per data point obtained using oocytes from three different frogs. \*,  $p < 0.05$ , compared with pH 7.5 by the Bonferroni method of multiple range testing.

**TABLE 1**  
Effect of pHo on Oatp1a1-, OATP1B1-, and OATP1B3-mediated TCA, E<sub>2</sub>17βG, and E3S uptake by transfected CHO cells  
Stably transfected CHO cells were incubated in uptake medium (96 mM choline chloride, 5.3 mM KCl, 1.1 mM KH<sub>2</sub>PO<sub>4</sub>, 0.8 mM MgSO<sub>4</sub>, 1.8 mM CaCl<sub>2</sub>, 11 mM glucose, and 50 mM HEPES) containing the desired substrate (10 μM [<sup>3</sup>H]TCA, 10 μM [<sup>3</sup>H]E<sub>2</sub>17βG, or 50 μM [<sup>3</sup>H]E3S) at 37°C for 15 min. Net uptake values are means ± S.D. from at least nine determinations per data point, obtained using cells from three different cultures.

	Oatp1a1	OATP1B1	OATP1B3
<i>pmol · mg protein<sup>-1</sup> · 15 min<sup>-1</sup></i>			
10 μM TCA			
pH 7.4	11 ± 2	38 ± 4	35 ± 3
pH 6.5	22 ± 2 <sup>a</sup>	28 ± 5 <sup>a</sup>	55 ± 7 <sup>a</sup>
10 μM E <sub>2</sub> 17βG			
pH 7.4	274 ± 20	203 ± 37	63 ± 8
pH 6.5	393 ± 15 <sup>a</sup>	135 ± 17 <sup>a</sup>	132 ± 19 <sup>a</sup>
50 μM E3S			
pH 7.4	1446 ± 71	456 ± 51	325 ± 35
pH 6.5	2212 ± 187 <sup>a</sup>	573 ± 73	479 ± 26 <sup>a</sup>

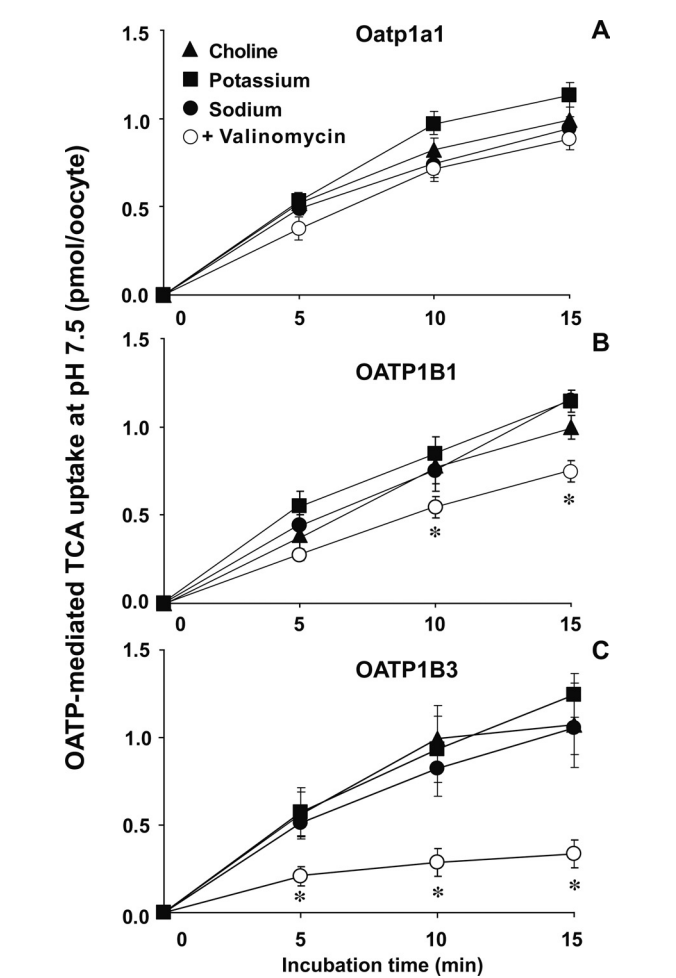
<sup>a</sup> *P* < 0.05, on comparing pH 6.5 with pH 7.4 by paired *t* test.



**Fig. 4.** Effect of CCCP or valinomycin on extracellular pH-induced changes in Oatp1a1-, OATP1B1-, and OATP1B3-mediated transport. *X. laevis* oocytes expressing Oatp1a1 (A), OATP1B1 (B), or OATP1B3 (C) were incubated in uptake medium containing 10 μM [<sup>3</sup>H]TCA alone (Control) with or without 50 μM CCCP or 10 μM valinomycin at 25°C for 1 h. Net TCA uptake was calculated by subtracting the amount of radioactivity measured in noninjected oocytes from that found in oocytes expressing Oatp/OATP. Net uptake values are means ± S.D. from at least 24 determinations per data point, obtained using oocytes from three different frogs. \*, *p* < 0.05, compared with Control by the Bonferroni method of multiple-range testing.

DilC<sub>1</sub>(5) were used to determine relative changes in pH<sub>i</sub> and ΔΨ, respectively. Transport experiments were carried out by incubating CHO cells in suspension with 100 μl of uptake medium containing 1 to 5 μM CGamF, 2 μM SNARF-4F, or 2 nM DilC<sub>1</sub>(5) at 37°C for 15 min. These concentrations were chosen based on preliminary studies (data not shown). Then, 900 μl of ice-cold uptake solution was added to stop the transport process and the intracellular fluorescence was determined immediately by flow cytometry. Photographs depicting the intracellular accumulation of CGamF after incubation with the substrate at a final concentration of 10 μM at 37°C for 15 min were obtained using a fluorescence microscope (Eclipse TE 2000-S; Nikon, Tokyo, Japan).

**Statistical Methods.** Results were expressed as means ± S.D. All studies were carried out using groups of 8 to 12 oocytes per data point, and the experiments were repeated using three different frogs. Regarding CHO cells, at least three different cultures were used per experiment. To calculate the statistical significance of the differences between groups, the paired *t* test or Bonferroni method were used, as appropriate.



**Fig. 5.** Effect of major cation replacement and valinomycin on Oatp1a1-, OATP1B1-, and OATP1B3-mediated transport. *X. laevis* oocytes expressing Oatp1a1 (A), OATP1B1 (B), or OATP1B3 (C) were incubated in uptake medium at pH 7.5, containing 10 μM [<sup>3</sup>H]TCA with or without 10 μM valinomycin at 25°C. In some experiments, 100 mM choline chloride was replaced by 100 mM sodium chloride or potassium chloride. Net TCA uptake was calculated by subtracting the amount of radioactivity measured in noninjected oocytes from that found in oocytes expressing Oatp/OATP incubated under the same conditions. Net uptake values are means ± S.D. from at least 24 determinations per data point, obtained using oocytes from three different frogs. \*, *p* < 0.05, compared with choline chloride medium by the Bonferroni method of multiple-range testing.

## Results

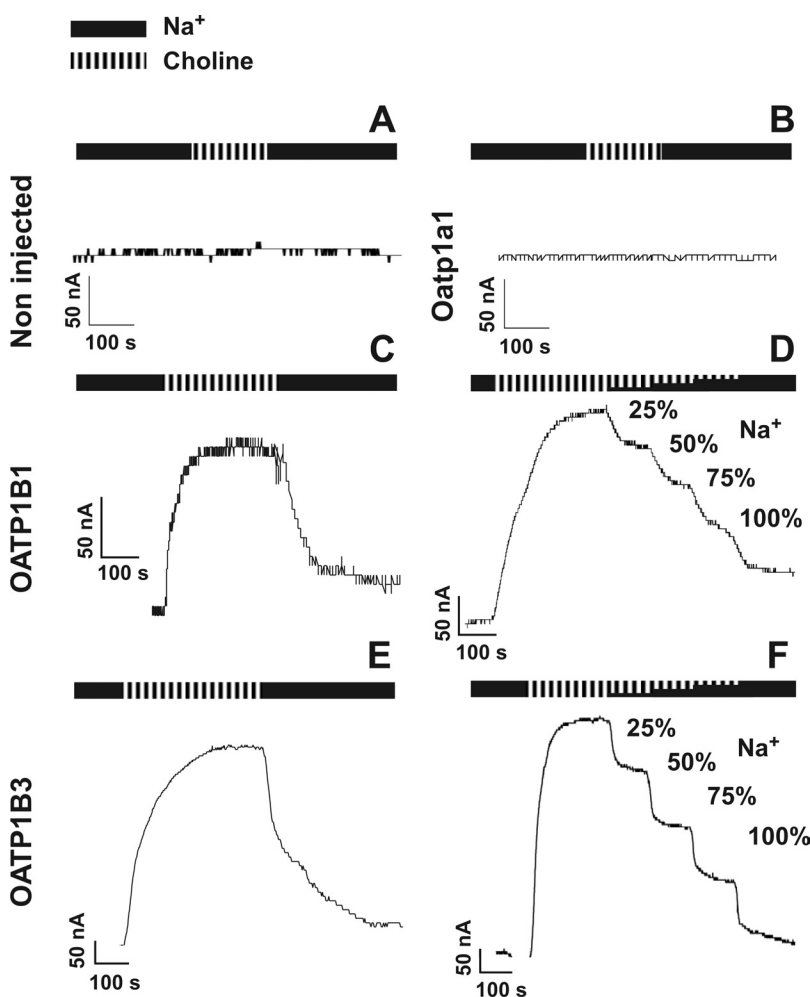
**Immunohistochemistry of Oocytes Expressing Oatp1a1, OATP1B1, or OATP1B3.** To assess the efficient and specific expression of rat Oatp1a1, human OATP1B1, and human OATP1B3 at the plasma membrane of *X. laevis* oocytes for functional studies, immunofluorescence analysis was carried out. Oatp1a1 (Fig. 1A), OATP1B1 (Fig. 1B), and OATP1B3 (Fig. 1C) were detected at the plasma membrane two days after injecting the appropriate cRNA. No fluorescence was observed using similar primary and secondary antibodies in noninjected oocytes. Figure 1D shows a noninjected oocyte incubated with antibodies used for Oatp1a1. Similar results were obtained for OATP1B1 and OATP1B3 (data not shown).

**Effect of pHo on Plasma Membrane Permeability.** Before studying pH dependence of Oatp/OATP-mediated transport, the sensitivity of the plasma membrane permeability of *X. laevis* oocytes to changes in pHo was investigated. The uptake by oocytes of two neutral compounds of different molecular weight—inulin and mannitol, which are assumed to have very low ability to diffuse across the plasma membrane—was determined. The values of the intracellular accumulation of these compounds at pHo varying from 7.5 to 3.5 were compared with those obtained in cells permeabilized by treatment with Triton X-100. In the range of pHo from 7.5 to 4.5, the uptake of inulin (Fig. 2A) and mannitol (Fig. 2B) was low and was not significantly changed as pHo was acid-

ified. In contrast, a marked increase was observed at pHo 3.5. These results indicated that changes in substrate uptake that were observed at pHo  $\geq 4.5$  and will be described below, cannot be attributed to modifications in plasma membrane permeability.

**Effect of pHo on the Carrier-Mediated TCA and E<sub>2</sub>17 $\beta$ G Uptake.** The instability of HEPES buffer at pHo 5.5 recommended the use of MES in part of the pHo range (7.5–4.5) investigated here. When Oatp1a1-expressing oocytes were incubated in the presence of TCA at pHo values of 6.5 a marked stimulation in TCA uptake was observed compared with pHo 7.5 (Fig. 3A). This increased slightly when pHo was decreased to 5.5 and 4.5. This effect was also observed when E<sub>2</sub>17 $\beta$ G, which has a pK<sub>a</sub> value 1.5 points higher than that of TCA, was used as a substrate (Fig. 3B). A different response of OATP1B1 to pHo was found. The net uptake of both TCA (Fig. 3C) and E<sub>2</sub>17 $\beta$ G (data not shown) decreased progressively as pHo became more acidic across the whole range assayed. The opposite behavior was observed for OATP1B3, which was progressively stimulated in the pHo range assayed (Fig. 3D).

To further investigate the effect of pHo on OATP-mediated transport, we tested the effect of changes in pHo on TCA, E<sub>2</sub>17 $\beta$ G, and E3S uptake by CHO expressing Oatp1a1, OATP1B1, or OATP1B3. Consistent with the observations in oocytes described above, pHo acidification from 7.4 to 6.5 significantly



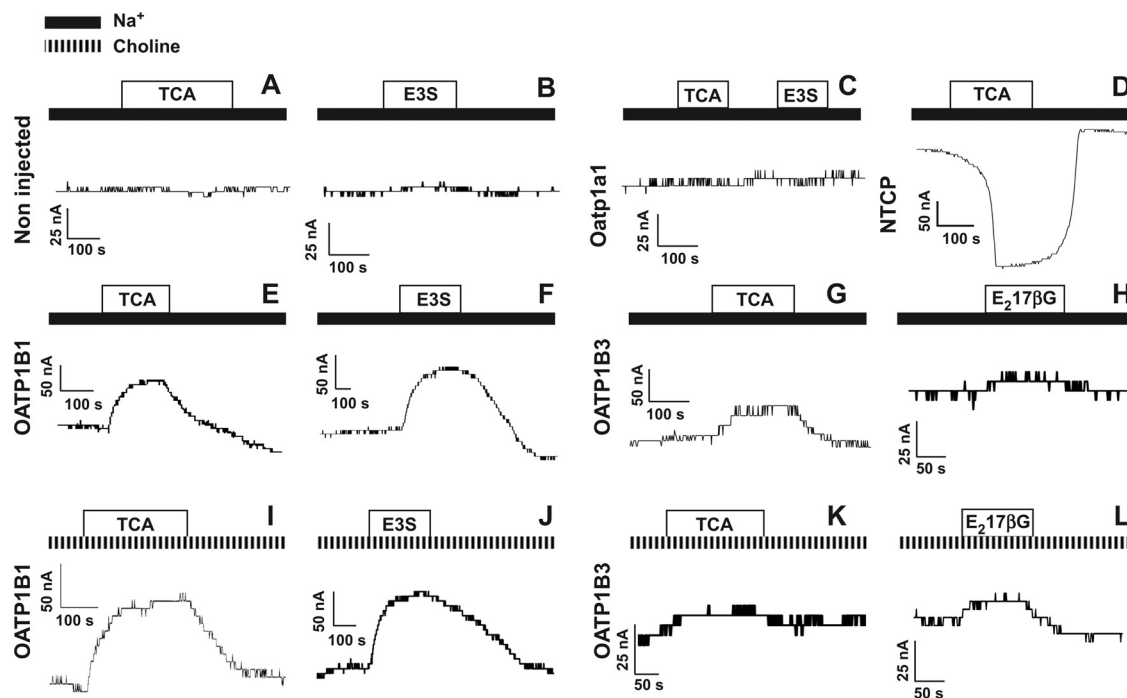
**Fig. 6.** Representative recordings of currents induced in *X. laevis* oocytes by replacement of sodium by choline. Noninjected oocytes (A) and oocytes expressing Oatp1a1 (B), OATP1B1 (C and D), or OATP1B3 (E and F) were perfused while the current was being recorded continuously. The perfused buffer contained either Na<sup>+</sup> (solid line), choline (dashed line), or Na<sup>+</sup> plus choline at the indicated proportions. Each recording was replicated in oocytes from three different batches.

stimulated TCA uptake by Oatp1a1 and OATP1B3, whereas the opposite effect was seen in OATP1B1-expressing cells (Table 1). Similar results for these three transporters were found when  $E_217\beta G$  was used as a substrate (Table 1). In contrast, when E3S uptake was measured different behavior was observed only for OATP1B1-mediated transport, which was not inhibited at acidic pHo (Table 1).

**Effect of CCCP, Valinomycin, and Cation Replacement on TCA Uptake by Oocytes.** To elucidate whether these changes in transport activity were related in part to the magnitude of  $\Delta\Psi$  and proton gradients across the plasma membrane, uptake measurements in *X. laevis* oocytes were carried out in the presence of the  $K^+$ -ionophore valinomycin or the protonophore CCCP for 1 h. At all values of pHo assayed, CCCP induced a marked inhibition in Oatp1a1-mediated TCA uptake (Fig. 4A), whereas valinomycin had no effect on Oatp1a1-mediated TCA uptake (Fig. 4A). In contrast, TCA uptake by OATP1B1-expressing oocytes was inhibited by CCCP at all pHo values assayed, whereas the presence of valinomycin slightly decreased the ability of OATP1B1 to take up the substrate at pHo > 4.5 (Fig. 4B). Oocytes expressing OATP1B3 were more sensitive to inhibition by valinomycin, whereas they were not affected by the presence of CCCP (Fig. 4C). Because long-time incubations may have affected pH<sub>i</sub> in addition to proton gradients, the effect of valinomycin and cation replacement was investigated by incubating the oocytes for a shorter time (5, 10, and 15 min). In these studies, valinomycin had no effect on Oatp1a1 (Fig. 5A). However, this ionophore induced a moderate and marked inhibition of TCA uptake in oocytes expressing OATP1B1 (Fig. 5B) and OATP1B3 (Fig. 5C), respectively. Moreover, the replacement of choline by sodium or

potassium had no significant effect on Oatp1a1-, OATP1B1-, or OATP1B3-mediated TCA (Fig. 5, A–C).

**Electrophysiology Experiments with Oatp/OATPs and Human NTCP.** Whereas Oatp1a1 has been believed to behave as an electroneutral transporter, the existence of electroneutral or electrogenic mechanisms in OATP1B1- and OATP1B3-mediated transport has not been elucidated. The fact that both were sensitive to valinomycin suggested that changes in  $\Delta\Psi$  may affect the function of these two carriers. This does not necessarily mean that the transport process is electrogenic, although this possibility would be consistent with these findings. To further investigate this question, we performed electrophysiology experiments using the two-electrode voltage-clamp method. Noninjected oocytes served as a negative control. As a positive control, oocytes expressing Oatp1a1 were used. Replacement of  $Na^+$  buffer by choline buffer had no effect on noninjected oocytes (Fig. 6A) or oocytes expressing Oatp1a1 (Fig. 6B). However, this maneuver induced an outward deflection of the current trace in oocytes expressing OATP1B1 (Fig. 6C) or OATP1B3 (Fig. 6E), being its magnitude markedly higher in the latter. Progressive replacement of choline by  $Na^+$  in the perfusion buffer caused a progressive reversal of this effect (Fig. 6, D and F). These results suggest that in the presence of  $Na^+$ , there is a leakage of  $Na^+$  through the transporter (OATP1B3 > OATP1B1), which is abolished by replacement of this cation by choline. The presence of substrate (either TCA or E3S) did not induce electric changes in noninjected oocytes (Fig. 7, A and B) or oocytes expressing Oatp1a1 (Fig. 7C). The latter is consistent with the concept that Oatp1a1 is an electroneutral anion transporter. The well known electrogenicity of the human bile acid transporter NTCP was used for comparison (Fig. 7D). NTCP-expressing oocytes were perfused



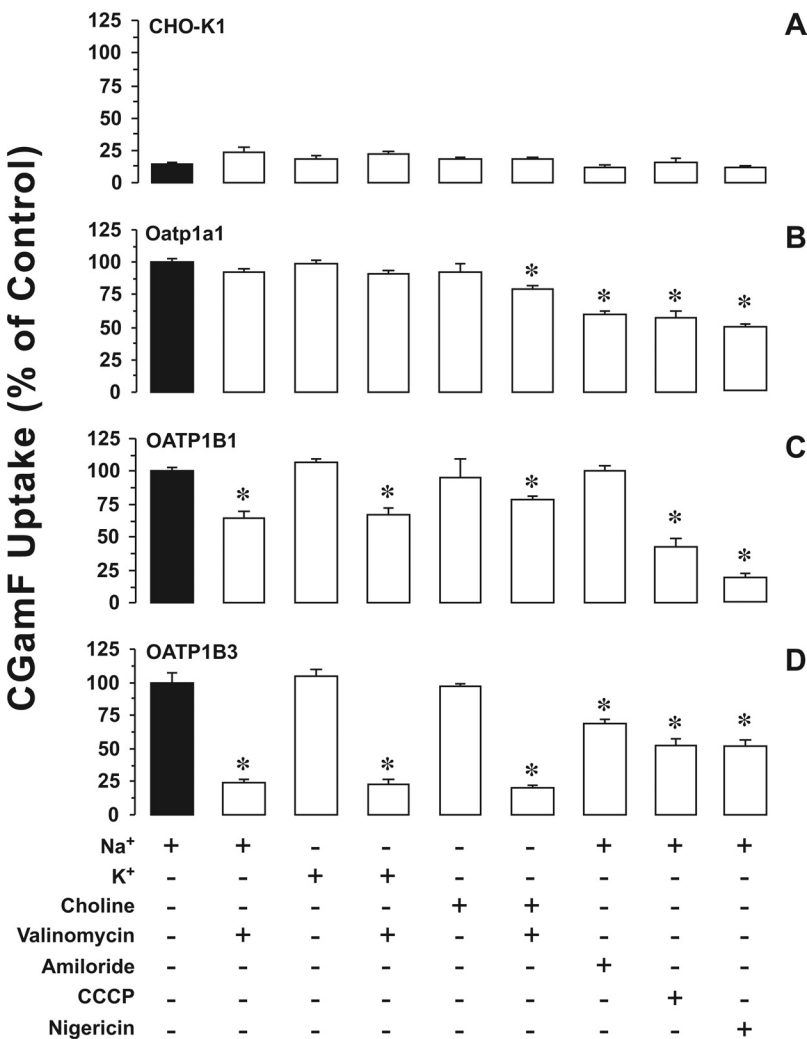
**Fig. 7.** Representative current recorded in *X. laevis* oocytes in the presence of substrates. Noninjected oocytes (A and B) and oocytes expressing rat Oatp1a1 (C), human NTCP (D), OATP1B1 (E, F, I, and J), or OATP1B3 (G, H, K, and L) were perfused with either  $Na^+$  (solid line) or choline (dashed line) containing buffer while the current was being recorded continuously. The addition of 100  $\mu M$  TCA, E3S, or  $E_217\beta G$  was followed by a washing period. Each recording was replicated in oocytes from three different batches.

with  $\text{Na}^+$  buffer and the current was recorded continuously. The perfusion with 100  $\mu\text{M}$  TCA induced an inward current that was seen as a downward deflection of the current trace. This corresponded to the injection of a negative current by the voltage-clamp equipment to maintain a holding  $\Delta\Psi$  at  $-50$  mV upon depolarization of the membrane, which was presumably due to the cotransport of  $\text{Na}^+$  together with TCA through NTCP. When the oocyte was washed with  $\text{Na}^+$  buffer, the original baseline level was recovered (Fig. 7D). In oocytes expressing OATP1B1 (Fig. 7E), perfusion with 100  $\mu\text{M}$  TCA resulted in an outward deflection of the current trace. A similar, although weaker, response was recorded in oocytes expressing OATP1B3 (Fig. 7G). This could be interpreted as being due to hyperpolarization of the plasma membrane associated to the net entrance of negative charge. To elucidate whether this effect could be induced by other OATP1B1/OATP1B3 substrates, 100  $\mu\text{M}$  E3S or E217 $\beta$ G was added to the perfusion medium. Both compounds induced hyperpolarization in oocytes expressing either OATP1B1 (Fig. 7F) or OATP1B3 (Fig. 7H). When these studies were repeated after replacing  $\text{Na}^+$  by choline the presence of substrate was also able to induce net entrance of negative charge in oocytes expressing either OATP1B1 (Fig. 7, I and J) or OATP1B3 (Fig. 7, K and L). Although in Fig. 7, E to L, representative fragments of recordings are shown, several manipulations were carried out using a single oocyte to confirm that the

magnitude of changes was not due to differences in the expression level of the transport protein among oocytes.

**Fluorescent Bile Acid (CGamF) Uptake by Transfected CHO Cells.** Using flow cytometry, some of the data obtained in the oocyte model were re-evaluated. First, the uptake of the fluorescent bile acid derivative CGamF by CHO cells was determined by measuring intracellular fluorescence at the indicated time points (Fig. 1, E and F) during 1 h of incubation. The expression of Oatp1a1, OATP1B1, or OATP1B3 markedly enhanced the ability of CHO cells to take up CGamF (Fig. 1, E and F). This was also observed when the cells were incubated under similar circumstances for 15 min and studied under the fluorescence microscope (Fig. 1, E and F, inset). The ability of OATP1B1 and OATP1B3 to take up CGamF has been reported previously (Gui et al., 2008; Annaert et al., 2010). To set up the appropriate incubation conditions, measurement of the time course of CGamF uptake was carried out (Fig. 1, E and F). Based on these results and in agreement with previous transport studies of bile acids in CHO cells (Marin et al., 2003), we selected 15-min incubation as an appropriate time to carry out further studies.

In agreement with the results described above obtained in oocytes and CHO cells using radiolabeled substrates, the uptake of CGamF by Oatp1a1-, OATP1B1-, or



**Fig. 8.** Uptake of CGamF by CHO cells expressing Oatp1a1-, OATP1B1-, or OATP1B3. Wild-type CHO (CHO-K1) cells (A) or CHO cells stably transfected with Oatp1a1 (B), OATP1B1 (C), or OATP1B3 (D) were incubated in different uptake media (5.3 mM KCl, 1.1 mM  $\text{KH}_2\text{PO}_4$ , 0.8 mM  $\text{MgSO}_4$ , 1.8 mM  $\text{CaCl}_2$ , 11 mM glucose, and 50 mM HEPES, pH 7.4) with or without 10  $\mu\text{M}$  valinomycin, 1 mM amiloride, 50  $\mu\text{M}$  CCCP, or 10  $\mu\text{M}$  nigericin. In addition, the medium contained either 96 mM NaCl, 96 mM KCl, or 96 mM choline. CGamF uptake was determined after incubating the cells at 37°C for 15 min in one of the media mentioned above to which 1  $\mu\text{M}$  (for OATP1B3) or 5  $\mu\text{M}$  (for Oatp1a1 and OATP1B1) of CGamF had been added. Results are expressed as percentages of the CGamF fluorescence of the cells incubated in NaCl medium alone. In the case of CHO-K1 cells, the control (100% value) was CGamF uptake by Oatp1a1 in NaCl medium. Uptake values are means  $\pm$  S.D. from at least nine determinations per data point, obtained using cells from three different cultures. \*,  $p < 0.05$ , compared with the CGamF uptake by controls by the Bonferroni method of multiple-range testing.

OATP1B3-expressing CHO cells was not affected by the replacement of choline by Na<sup>+</sup> or K<sup>+</sup> (Fig. 8). The fluorescent probe SNARF-4F ester freely diffuses inside the cell, where its fluorescence increases as pHi becomes more basic. Moreover, by determining intracellular DiIC<sub>1</sub>(5), the changes in ΔΨ that took place in CHO cells were followed. Because marked changes in pH affect the fluorescence spectra of CGamF (data not shown), this could not be used to study the influence of a broad range of pH in this model. However, it was still possible to follow changes in CGamF uptake in the moderate range of variation in pHi induced by the maneuvers carried out in these experiments (Fig. 9). Thus, the presence of CCCP or nigericin induced an intracellular alkalinization (Fig. 9), with no significant change in ΔΨ (Fig. 10). This resulted in a decreased CGamF uptake by Oatp1a1-, OATP1B1-, and OATP1B3-expressing cells (Fig. 8).

We used amiloride to block Na<sup>+</sup>/H<sup>+</sup> exchange and induce intracellular acidification, which was confirmed by decreased amounts of SNARF-4F fluorescence in the cells (Fig. 9), with no changes in ΔΨ (Fig. 10). This reduced CGamF uptake by Oatp1a1- and OATP1B3-expressing cells but had no significant effect on OATP1B1-mediated CGamF uptake (Fig. 8).

Valinomycin resulted in a significant increase in DiIC<sub>1</sub>(5) fluorescence, which, considering the cationic nature of the probe, was interpreted as being due to plasma membrane

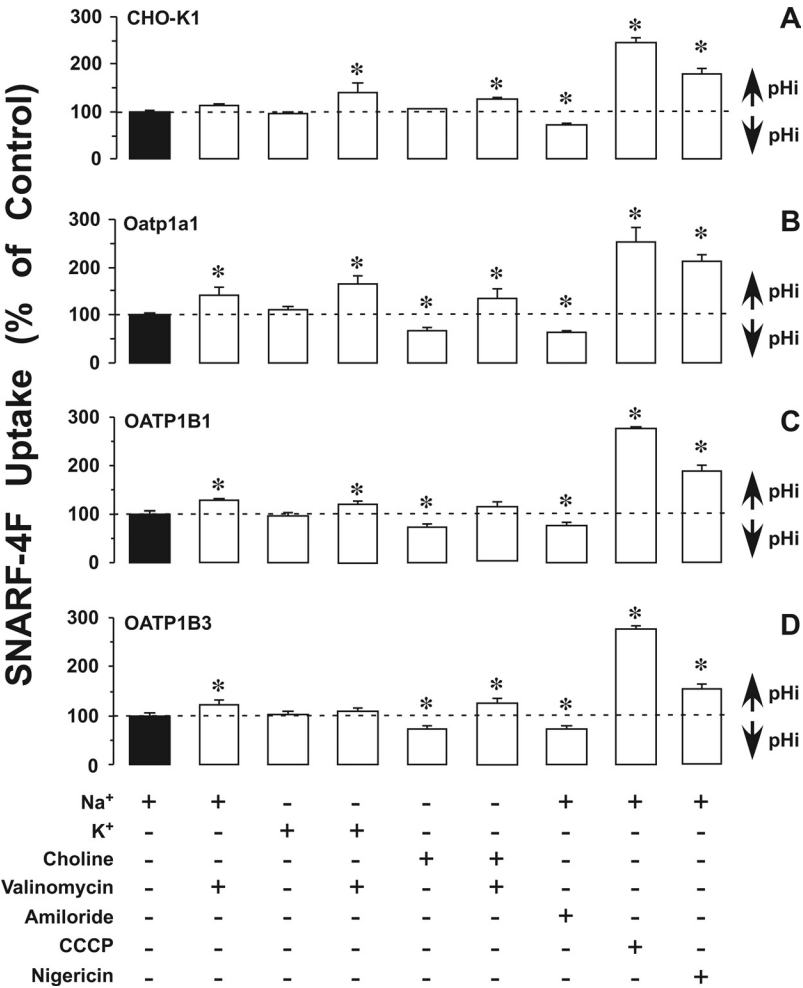
hyperpolarization (Fig. 10). This had no effect on CGamF uptake by Oatp1a1 (Fig. 8) but inhibited that mediated by OATP1B1 (moderately) and OATP1B3 (markedly) (Fig. 8).

Discussion

The dependence of OATP-mediated transport on pHo has been investigated previously (Leuthold et al., 2009). The present study further elucidates this issue with regard to two important transporters, OATP1B1 and OATP1B3, involved in the uptake of many endogenous and xenobiotic compounds by the liver. For comparative purposes, in this study we included the well known rat isoform Oatp1a1. Human NTCP was also included in electrophysiological studies as a positive control for an electrogenic bile acid transporter (Weinman, 1997).

Our results suggest that changes in pHo can markedly affect the transport ability of OATP1B1 and OATP1B3. This occurs at pHo values close to those present in normal healthy tissue or in certain pathological environments; that is, at values markedly higher than those able to affect the physical characteristics of plasma membrane and hence increase permeability in a nonselective manner. At least in oocytes, this occurred at pHo <4.5 and was accompanied by enhanced incorporation of mannitol and inulin into these cells.

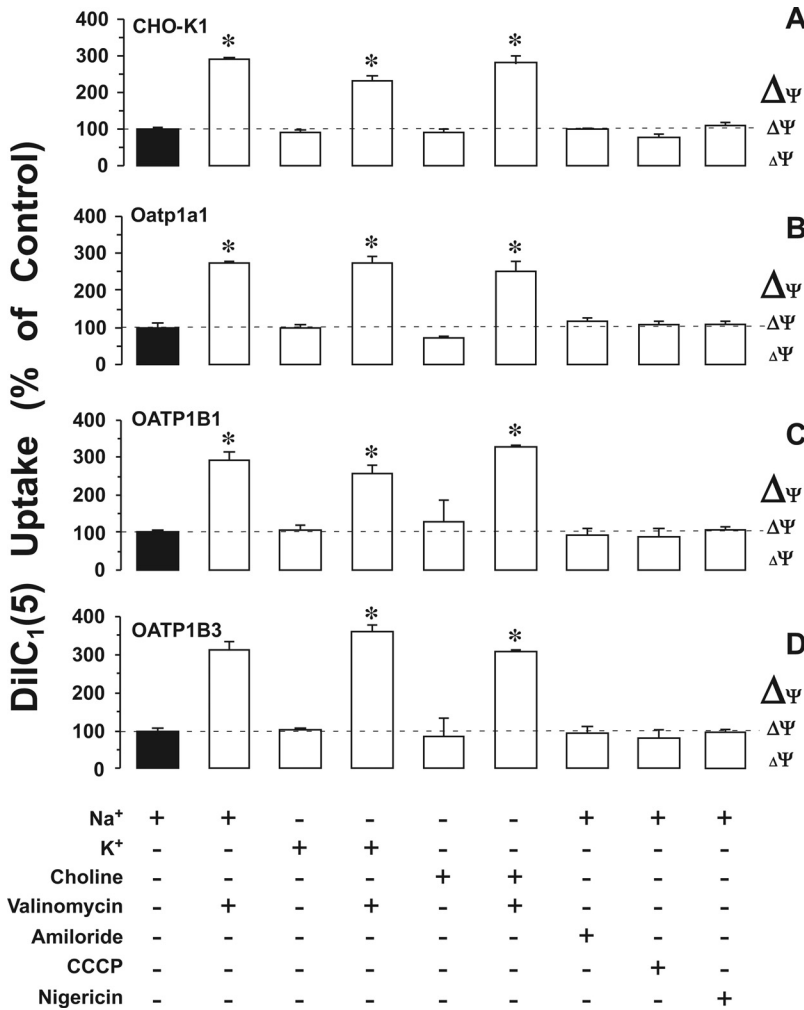
At pHo >4.5, the pattern of response to changes in pHo was specific for each transporter but, in each case, similar for two different substrates: TCA and E<sub>2</sub>17βG. This response



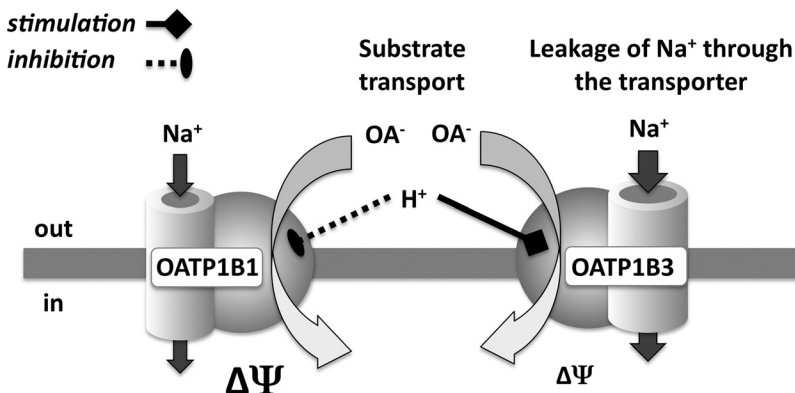
**Fig. 9.** Uptake of SNARF-4F by CHO cells expressing Oatp1a1-, OATP1B1-, or OATP1B3. Wild-type CHO (CHO-K1) cells (A) or CHO cells stably transfected with Oatp1a1 (B), OATP1B1 (C), or OATP1B3 (D) were incubated in different uptake media (5.3 mM KCl, 1.1 mM KH<sub>2</sub>PO<sub>4</sub>, 0.8 mM MgSO<sub>4</sub>, 1.8 mM CaCl<sub>2</sub>, 11 mM glucose, and 50 mM HEPES, pH 7.4) with or without 10 μM valinomycin, 1 mM amiloride, 50 μM CCCP, or 10 μM nigericin. In addition, the media contained either 96 mM NaCl, 96 mM KCl, or 96 mM choline. SNARF-4F uptake was determined after incubating the cells at 37°C for 15 min in one of the media mentioned above to which 2 μM SNARF-4F ester had been added. Results are expressed as percentages of the SNARF-4F fluorescence of the cells incubated in NaCl medium alone. Uptake values are means ± S.D. from at least nine determinations per data point, obtained using cells from three different cultures. \*, *p* < 0.05, compared with the SNARF-4F uptake by controls by the Bonferroni method of multiple-range testing.

was consistent with pH-dependent protonation of certain domains in these proteins, which has been suggested to affect their transport activity (Leuthold et al., 2009). The results of the present study are in part consistent with those findings. Thus, the OATP1B3-mediated uptake of several compounds, such as prostaglandin E<sub>2</sub> and L-thyroxine, into *X. laevis* oocytes has been reported to be higher at pH 6.5 than at pH 8.0 (Leuthold et al., 2009). In contrast, OATP1B1-mediated transport was not affected by this change in pH (Leuthold et al., 2009). In the present study, by using a broader range of pH<sub>o</sub> and carrying out several manipulations, sensitivity of OATP1B1 to pH<sub>o</sub> was found. However, the response to pH<sub>o</sub>

acidification was opposite for OATP1B1 (decrease) and OATP1B3 (increase) activity. The reason for this apparent controversy may be due in part to the complexity of the response of these transporters to changes in pH<sub>o</sub>. Previous studies have suggested that the OATP1B1-mediated transport of certain compounds, such as E3S, probably involved the interaction of these substrates with two sites of the carrier, with high ( $K_m = 0.23 \mu\text{M}$ ) and low ( $K_m = 45 \mu\text{M}$ ) affinity. However, most of the identified OATP1B1 substrates, such as bile acids and E<sub>2</sub>17 $\beta$ G, are able to bind only to the high-affinity site (Noe et al., 2007). These two sites may have different pH<sub>o</sub> sensitivities (Leuthold et al., 2009).



**Fig. 10.** Uptake of DilC<sub>1</sub>(5) by CHO (CHO-K1) cells expressing Oatp1a1-, OATP1B1-, or OATP1B3. Wild-type CHO cells (A) or CHO cells stably transfected with Oatp1a1 (B), OATP1B1 (C), or OATP1B3 (D) were incubated in different uptake media (5.3 mM KCl, 1.1 mM KH<sub>2</sub>PO<sub>4</sub>, 0.8 mM MgSO<sub>4</sub>, 1.8 mM CaCl<sub>2</sub>, 11 mM glucose, and 50 mM HEPES, pH 7.4) with or without 10  $\mu\text{M}$  valinomycin, 1 mM amiloride, 50  $\mu\text{M}$  CCCP, or 10  $\mu\text{M}$  nigericin. In addition, the media contained either 96 mM NaCl, 96 mM KCl, or 96 mM choline. DilC<sub>1</sub>(5) uptake was determined after incubating the cells at 37°C for 15 min in one of the media mentioned above to which 2 nM DilC<sub>1</sub>(5) had been added. Results are expressed as percentages of the DilC<sub>1</sub>(5) fluorescence of the cells incubated in NaCl medium alone. Uptake values are means  $\pm$  S.D. from at least nine determinations per data point, obtained using cells from three different cultures. \*,  $p < 0.05$ , compared with the DilC<sub>1</sub>(5) uptake by controls by the Bonferroni method of multiple-range testing.  $\Delta\Psi$ , plasma membrane potential.



**Fig. 11.** Scheme of the proposed models for OATP1B1 and OATP1B3 regarding electrogenicity and sensitivity to pH. OA<sup>-</sup>, organic anion.

Indeed, in the present study, the effect of changes in pHo on OATP1B1-mediated transport was clearly different for TCA and E<sub>2</sub>17βG compared with E3S. These results partially agree with those reported by Mahagita et al. (Mahagita et al., 2007) who also observed a lack of pHo dependence for E3S transport by OATP1B1 and OATP1B3. In the present study, the transport of E3S by OATP1B3, but not by OATP1B1, was found to be sensitive to pHo.

Next, we investigated whether, in addition to the hypothesized pHo-dependent changes in the protein (Leuthold et al., 2009), the activity of these transporters could also be affected by changes in the magnitude and direction of the transmembrane proton (or inversely directed OH<sup>-</sup>) fluxes. When the protonophore CCCP was added to the incubation media of either oocytes or CHO cells, the response of Oatp1a1 (activation by pHo acidification) was markedly reduced. This was probably due to changes in proton flux rather than to voltage-dependent processes because, as measured in CHO cells at pHo 7.4, CCCP and nigericin induced intracellular alkalization without significantly affecting ΔΨ. In this experimental model, all three transporters, Oatp1a1, OATP1B1, and OATP1B3, were negatively affected by pH<sub>i</sub> displacement in both directions, either acidification or alkalization. When valinomycin was added to oocytes or mammalian cells, an enhanced permeability to K<sup>+</sup> induced by this ionophore was expected. Consequently, after an initial efflux of K<sup>+</sup>, the plasma membrane presumably reaches a steady state of decreased electric resistance with enhanced difficulty for the cell to maintain ΔΨ, hence causing plasma membrane depolarization. This change had no effect on Oatp1a1-mediated transport. These results and electrophysiological recordings of Oatp1a1-mediated TCA transport further supported the concept previously suggested by others (Satlin et al., 1997; Li et al., 1998) that rat Oatp1a1 behaves as an electroneutral anion exchanger that is sensitive to pHo but unaffected by changes in ΔΨ.

The results of the present study indicate that OATP1B1 shares some of the functional characteristics described previously for Oatp1a1 (Hagenbuch and Meier, 2003; Briz et al., 2006) but has its own peculiarities. Thus, in contrast to Oatp1a1, the activity of OATP1B1 decreased as pHo became more acidic. This is consistent with different structural changes in the protein induced by extracellular acidity. Moreover, valinomycin inhibited OATP1B1-mediated transport, which suggested that OATP1B1 is sensitive to changes in ΔΨ. Moreover, electrophysiological experiments showed that substrate transport induced a strong current through OATP1B1. Compared with the current accompanying TCA transport through NTCP, the current recorded in oocytes expressing OATP1B1 and perfused with TCA, E3S, or E<sub>2</sub>17βG was of opposite direction. Because NTCP inwardly transports one anion (bile acid) per two cations (sodium), the result is a net influx of positive charge. This suggests that an electrogenic influx of anions (or efflux of cations) accompanies substrate uptake through OATP1B1. The simplest explanation for these results (Fig. 11) is that the influx of negative charge would be due to transport of the anionic substrate through OATP1B1. Surprisingly, we have observed that even in the absence of substrate, and hence of transport process (basal condition), there exists an influx of Na<sup>+</sup> through OATP1B1, which disappears when Na<sup>+</sup> was replaced by choline. These findings, together with the lack of

effect on substrate uptake when Na<sup>+</sup> was replaced by K<sup>+</sup>, and the similarity of the electrogenic effect associated to substrate transport in the presence of Na<sup>+</sup> or choline, are consistent with the existence of a leakage of Na<sup>+</sup> through the transporter, not coupled to the transport of the substrate (Fig. 11).

The responses of OATP1B3 were in some cases similar to those observed for Oatp1a1 (activation by extracellular acidification) and in some cases to OATP1B1 (electrogenicity). Despite the marked activation of OATP1B3-mediated transport when pHo was acidified, incubation in the presence of a protonophore had no significant effect (oocytes) or the effect was lower than that observed for OATP1B1 (CHO cells). This suggests that transmembrane proton flux and/or changes in intracellular pH have less effect on OATP1B3 than on OATP1B1 activity, as was confirmed using CHO cells (Figs. 8 and 9). Nevertheless, probably as a result of conformational changes (Leuthold et al., 2009), OATP1B3 activity is highly sensitive to environmental acidity, which may explain previous controversial results regarding OATP1B3 activation by glutathione, which were misinterpreted as being due to glutathione itself (Briz et al., 2006). However, OATP1B3 activation was abolished when glutathione-induced changes in pHo were controlled (Mahagita et al., 2007). In contrast to the lower sensitivity of OATP1B3 to proton movements through the plasma membrane, OATP1B3 is markedly affected by valinomycin, suggesting that this transporter was very sensitive to changes in ΔΨ. Similarly to OATP1B1, OATP1B3-mediated transport is accompanied of a current across the plasma membrane, although the magnitude was markedly lower than those recorded during OATP1B1-mediated transport. As suggested for OATP1B1, the simplest interpretation of these results would be that both proteins would behave as electrogenic carrier-mediated diffusion pathways for the uptake of organic anions (Fig. 11). However, the possibility that both transporters are anion exchangers with a stoichiometry X:1, where X ≥ 2, cannot be ruled out. An important characteristic of OATP1B3 shared with OATP1B1 but absent in Oatp1a1 is the existence of a basal leakage of Na<sup>+</sup> through the transporter. This seems to be of markedly higher magnitude in OATP1B3 than in OATP1B1.

Other transporters of organic anions, such as the folate transporter PCFT (*SLC46A1*), which mediates intestinal folate absorption and folate transport into the cells, also depend on electrogenic mechanisms and are highly dependent on pHo, being more active at pHo values of approximately 4.5 to 5.5 and very poor at pHo values of approximately ≥7.0 (Unal et al., 2009). This feature has been considered as being of particular importance for the delivery of antifolate analogs, such as pemetrexed, within the acidic milieu of solid tumors (Zhao and Goldman, 2007). Accordingly, because OATP1B1 and OATP1B3 are able to transport some antineoplastic drugs, such as irinotecan and methotrexate, respectively (Abe et al., 2001; Nozawa et al., 2005), it would be expected that the activation/inactivation of these transporters at acidic pH could affect the uptake of these drugs by tumor cells.

In conclusion, the results of the present study suggest that OATP1B1 and OATP1B3 are electrogenic transporters whose activity may be strongly affected by ΔΨ and local pH. This may determine the efficacy of drug uptake in certain healthy territories (e.g., the gastrointestinal tract, lung, and

kidney) and pathological tissues (e.g., the inner region of many tumors, inflammation foci) where the extracellular pH is displaced toward acidity from the generally normal value of 7.2 to 7.4 (Grinstein et al., 1991; Kraus and Wolf, 1996).

# Acknowledgments

We thank Drs. Peter Meier, Bruno Steiger, and Bruno Hagenbuch for the generous supply of antibodies, recombinant plasmids, and stably transfected CHO cells. Thanks are also due to L. Muñoz, J. F. Martin, J. Villoria, and N. Gonzalez for care of the animals. We gratefully acknowledge revision of the English spelling, grammar, and style of the manuscript by N. Skinner.

# Authorship Contributions

**Participated in research design:** Martinez-Becerra, Briz, Romero, Macias, Perez, Sancho-Mateo, Lostao, Fernandez-Abalos, and Marin.

**Conducted experiments:** Martinez-Becerra, Briz, Romero, Macias, Perez, Sancho-Mateo, Lostao, Fernandez-Abalos, and Marin.

**Contributed new reagents or analytic tools:** Martinez-Becerra, Briz, Romero, Macias, Perez, Sancho-Mateo, Lostao, Fernandez-Abalos, and Marin.

**Performed data analysis:** Martinez-Becerra, Briz, Romero, Macias, Perez, Sancho-Mateo, Lostao, Fernandez-Abalos, and Marin.

**Wrote or contributed to the writing of the manuscript:** Martinez-Becerra, Briz, Romero, Macias, Perez, Sancho-Mateo, Lostao, Fernandez-Abalos, and Marin.

# References

- Abe T, Unno M, Onogawa T, Tokui T, Kondo TN, Nakagomi R, Adachi H, Fujiwara K, Okabe M, Suzuki T, et al. (2001) LST-2, a human liver-specific organic anion transporter, determines methotrexate sensitivity in gastrointestinal cancers. *Gastroenterology* **120**:1689–1699.
- Ahya SN, Jose Soler M, Levitsky J, and Batlle D (2006) Acid-base and potassium disorders in liver disease. *Semin Nephrol* **26**:466–470.
- Annaert P, Ye ZW, Stieger B, and Augustijns P (2010) Interaction of HIV protease inhibitors with OATP1B1, 1B3, and 2B1. *Xenobiotica* **40**:163–176.
- Ballatori N, Hammond CL, Cunningham JB, Krance SM, and Marchan R (2005) Molecular mechanisms of reduced glutathione transport: role of the MRP/CFTR/ABCC and OATP/SLC21A families of membrane proteins. *Toxicol Appl Pharmacol* **204**:238–255.
- Briz O, Romero MR, Martinez-Becerra P, Macias RI, Perez MJ, Jimenez F, San Martin FG, and Marin JJ (2006) OATP8/1B3-mediated cotransport of bile acids and glutathione: an export pathway for organic anions from hepatocytes? *J Biol Chem* **281**:30326–30335.
- Briz O, Serrano MA, Rebollo N, Hagenbuch B, Meier PJ, Koepsell H, and Marin JJ (2002) 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**:853–860.
- Forsythe SM and Schmidt GA (2000) Sodium bicarbonate for the treatment of lactic acidosis. *Chest* **117**:260–267.
- Grinstein S, Swallow CJ, and Rotstein OD (1991) Regulation of cytoplasmic pH in phagocytic cell function and dysfunction. *Clin Biochem* **24**:241–247.
- Gui C, Miao Y, Thompson L, Wahlgren B, Mock M, Stieger B, and Hagenbuch B (2008) Effect of pregnane X receptor ligands on transport mediated by human OATP1B1 and OATP1B3. *Eur J Pharmacol* **584**:57–65.
- Hagenbuch B and Meier PJ (2003) The superfamily of organic anion transporting polypeptides. *Biochim Biophys Acta* **1609**:1–18.
- Hagenbuch B, Stieger B, Foguet M, Lubbert H, and Meier PJ (1991) Functional expression cloning and characterization of the hepatocyte Na<sup>+</sup>/bile acid cotransport system. *Proc Natl Acad Sci USA* **88**:10629–10633.
- Institute of Laboratory Animal Resources (1996) *Guide for the Care and Use of Laboratory Animals*, 7th ed. Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council, Washington DC.
- Kanai N, Lu R, Bao Y, Wolkoff AW and Schuster VL (1996) Transient expression of oatp organic anion transporter in mammalian cells: identification of candidate substrates. *Am J Physiol* **270**:F319–F325.
- Koepsell H and Endou H (2004) The SLC22 drug transporter family. *Pflugers Arch* **447**:666–676.
- Kraus M and Wolf B (1996) Implications of acidic tumor microenvironment for neoplastic growth and cancer treatment: a computer analysis. *Tumour Biol* **17**:133–154.
- Kullak-Ublick GA, Ismail MG, Stieger B, Landmann L, Huber R, Pizzagalli F,

- Fattinger K, Meier PJ, and Hagenbuch B (2001) Organic anion-transporting polypeptide B (OATP-B) and its functional comparison with three other OATPs of human liver. *Gastroenterology* **120**:525–533.
- Kullak-Ublick GA, Stieger B, and Meier PJ (2004) Enterohepatic bile salt transporters in normal physiology and liver disease. *Gastroenterology* **126**:322–342.
- Larrayoz IM, Fernandez-Nistal A, Garces A, Gorraitz E, and Lostao MP (2006) Characterization of the rat Na<sup>+</sup>/nucleoside cotransporter 2 and transport of nucleoside-derived drugs using electrophysiological methods. *Am J Physiol Cell Physiol* **291**:C1395–C1404.
- Lee TK, Hammond CL, and Ballatori N (2001) Intracellular glutathione regulates taurocholate transport in HepG2 cells. *Toxicol Appl Pharmacol* **174**:207–215.
- Leuthold S, Hagenbuch B, Mohebbi N, Wagner CA, Meier PJ, and Stieger B (2009) Mechanisms of pH-gradient driven transport mediated by organic anion polypeptide transporters. *Am J Physiol Cell Physiol* **296**:C570–C582.
- Li L, Lee TK, Meier PJ, and Ballatori N (1998) Identification of glutathione as a driving force and leukotriene C4 as a substrate for Oatp1, the hepatic sinusoidal organic solute transporter. *J Biol Chem* **273**:16184–16191.
- Li L, Meier PJ, and Ballatori N (2000) Oatp2 mediates bidirectional organic solute transport: a role for intracellular glutathione. *Mol Pharmacol* **58**:335–340.
- Mahagita C, Grassl SM, Piyachaturawat P, and Ballatori N (2007) Human organic anion transporter 1B1 and 1B3 function as bidirectional carriers and do not mediate GSH-bile acid cotransport. *Am J Physiol Gastrointest Liver Physiol* **293**:G271–G278.
- Marin JJ, Briz O, Perez MJ, Romero MR, and Monte MJ (2009) Hepatobiliary transporters in the pharmacology and toxicology of anticancer drugs. *Front Biosci* **14**:4257–4280.
- Marin JJ, Mangas D, Martinez-Diez MC, El-Mir MY, Briz O and Serrano MA (2003) Sensitivity of bile acid transport by organic anion-transporting polypeptides to intracellular pH. *Biochim Biophys Acta* **1611**(1–2):249–257.
- Markwell MA, Haas SM, Bieber LL, and Tolbert NE (1978) A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* **87**:206–210.
- Meier PJ and Stieger B (2002) Bile salt transporters. *Annu Rev Physiol* **64**:635–661.
- Meier-Abt F, Mokrab Y, and Mizuguchi K (2005) Organic anion transporting polypeptides of the OATP/SLCO superfamily: identification of new members in non-mammalian species, comparative modeling and a potential transport mode. *J Membr Biol* **208**:213–227.
- Nakanishi T, Doyle LA, Hassel B, Wei Y, Bauer KS, Wu S, Pumphlin DW, Fang HB, and Ross DD (2003) Functional characterization of human breast cancer resistance protein (BCRP, ABCG2) expressed in the oocytes of *Xenopus laevis*. *Mol Pharmacol* **64**:1452–1462.
- Noe J, Portmann R, Brun ME, and Funk C (2007) Substrate-dependent drug-drug interactions between gemfibrozil, fluvastatin and other organic anion-transporting peptide (OATP) substrates on OATP1B1, OATP2B1, and OATP1B3. *Drug Metab Dispos* **35**:1308–1314.
- Nozawa T, Imai K, Nezu J, Tsuji A, and Tamai I (2004) Functional characterization of pH-sensitive organic anion transporting polypeptide OATP-B in human. *J Pharmacol Exp Ther* **308**:438–445.
- Nozawa T, Minami H, Sugiyama S, Tsuji A, and Tamai I (2005) Role of organic anion transporter OATP1B1 (OATP-C) in hepatic uptake of irinotecan and its active metabolite, 7-ethyl-10-hydroxycamptothecin: in vitro evidence and effect of single nucleotide polymorphisms. *Drug Metab Dispos* **33**:434–439.
- Palermo DP, DeGraaf ME, Marotti KR, Rehberg E, and Post LE (1991) Production of analytical quantities of recombinant proteins in Chinese hamster ovary cells using sodium butyrate to elevate gene expression. *J Biotechnol* **19**:35–47.
- Sai Y and Tsuji A (2004) Transporter-mediated drug delivery: recent progress and experimental approaches. *Drug Discov Today* **9**:712–720.
- Satlin LM, Amin V, and Wolkoff AW (1997) Organic anion transporting polypeptide mediates organic anion/HCO<sub>3</sub><sup>−</sup> exchange. *J Biol Chem* **272**:26340–26345.
- Sherman IA and Fisher MM (1986) Hepatic transport of fluorescent molecules: in vivo studies using intravital TV microscopy. *Hepatology* **6**:444–449.
- Shi X, Bai S, Ford AC, Burk RD, Jacquemin E, Hagenbuch B, Meier PJ, and Wolkoff AW (1995) Stable inducible expression of a functional rat liver organic anion transport protein in HeLa cells. *J Biol Chem* **270**:25591–25595.
- Suzuki H and Sugiyama Y (2000) Transport of drugs across the hepatic sinusoidal membrane: sinusoidal drug influx and efflux in the liver. *Semin Liver Dis* **20**:251–263.
- Unal ES, Zhao R, Chang MH, Fiser A, Romero MF, and Goldman ID (2009) The functional roles of the His247 and His281 residues in folate and proton translocation mediated by the human proton-coupled folate transporter SLC46A1. *J Biol Chem* **284**:17846–17857.
- Weinman SA (1997) Electrogenicity of Na<sup>+</sup>-coupled bile acid transporters. *Yale J Biol Med* **70**:331–340.
- Zhao R and Goldman ID (2007) The molecular identity and characterization of a proton-coupled folate transporter-PCFT; biological ramifications and impact on the activity of pemetrexed. *Cancer Metastasis Rev* **26**:129–139.

**Address correspondence to:** Dr. Jose J. G. Marin Department of Physiology and Pharmacology Campus Miguel de Unamuno, E.D. S-09 37007-Salamanca, Spain. E-mail: jggmarin@usal.es