

Characterization of an organic anion transport system in a placental cell line

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Zhou, Fanfan, Kunihiro Tanaka, Michael J. Soares, and Guofeng You. Characterization of an organic anion transport system in a placental cell line. *Am J Physiol Endocrinol Metab* 285: E1103–E1109, 2003. First published August 5, 2003; 10.1152/ajpendo.00182.2003.—Transporters within the placenta play a crucial role in the distribution of nutrients and xenobiotics across the maternal-fetal interface. An organic anion transport system was identified on the apical membrane of the rat placenta cell line HRP-1, a model for the placenta barrier. The apical uptake of ³H-labeled organic anion estrone sulfate in HRP-1 cells was saturable ($K_m = 4.67 \mu\text{M}$), temperature and Na^+ dependent, Li^+ tolerant, and pH sensitive. The substrate specificity of the transport system includes various steroid sulfates, such as β -estradiol 3,17-disulfate, 17 β -estradiol 3-sulfate, and dehydroepiandrosterone 3-sulfate (DHEAS) but does not include taurocholate, *p*-aminohippuric acid (PAH), and tetraethylammonium. Preincubation of HRP-1 cells with 8-bromo-cAMP (a cAMP analog) and forskolin (an adenyl cyclase activator) acutely stimulated the apical transport activity. This stimulation was further enhanced in the presence of IBMX (a phosphodiesterase inhibitor). Together these data show that the apical membrane of HRP-1 cells expresses an organic anion transport system that is regulated by cellular cAMP levels. This transport system appears to be different from the known taurocholate-transporting organic anion-transporting polypeptides and PAH-transporting organic anion transporters, both of which also mediate the transport of estrone sulfate and DHEAS.

placenta; organic anion; transporters

THE PLACENTA forms the sole structural barrier between the mother and the developing fetus and performs many functions that are essential for normal fetal development. One of the major functions of the placenta is to mediate the transfer of nutrients from the mother to the fetus and to eliminate metabolic waste products from the fetus (2, 9, 16). This function is facilitated by the polarized expression of various transporters in the maternal-facing brush border membrane and the fetal-facing basal membrane of the placenta epithelium. The specificity of these transporters is, however, not limited to their physiological substrates. Xenobiotics bearing structural similarity to the physi-

ological substrates have the potential to be recognized by these transporters. These compounds include therapeutic agents and environmental toxins. Therefore, the transporters expressed in the placenta crucially influence the distribution of these xenobiotics across the maternal-fetal interface (2, 9, 16).

Many xenobiotics are organic anions or are metabolized to organic anions. Their absorption and/or elimination into and/or from the body are handled by several groups of organic anion transporters (OATs) (11, 34). Although the roles of these transporters in other tissues such as kidney and liver have been extensively studied, their roles in placenta have just begun to be explored. The placenta is known to transport various organic anions such as steroid sulfates, bile acids, and thyroid hormones (1, 24, 29). Recently, several OATs have been identified in the placenta. At gene level, the mRNAs for organic anion transporting polypeptides (OATPs), Oatp1, Oatp2, Oatp3, Oatp4, Oatp5, Oatp9 and Oatp12, have been detected, with various degrees of abundance, in rat placenta (19). However, whether any of these genes produces a functional transporter in this organ is not known. At the protein level, OAT4 and OATP-B have been immunohistochemically localized to the basolateral membranes of human placenta and human placenta-derived BeWo cells (30, 31). This basolateral localization is consistent with the functional expression of a transport system in basal syncytiotrophoblast membrane vesicles. OATP-E protein has been localized to the apical membrane of human placenta by the immunohistochemical approach (26). However, no functional data correlating with this apical expression are yet available. Furthermore, no information is available for the placental regulation of organic anion transport. In the present study, we identified, by functional analyses, an organic anion transport system on the apical membrane of the placenta cell line HRP-1. We also dissected the transport mechanism and investigated its intracellular regulatory pathways.

The HRP-1 cell line is derived from normal rat placenta and appears morphologically similar to, and retains characteristic expression of, cellular markers of labyrinthine trophoblast cells, in which the bulk of

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maternal-fetal nutrient/waste transfer occurs (13, 14, 20). This cell line has proved to be a useful in vitro model system to study function and regulation of several transporters, including glutamate transporters (22), fatty acid transporters (17), and a facilitative glucose transporter (5). Therefore, we have chosen this cell line for the present studies.

MATERIALS AND METHODS

Materials. [^3H]estrone sulfate, [^3H]dehydroepiandrosterone 3-sulfate (DHEAS), and [^{14}C]taurocholate were purchased from Perkin Elmer Life Science. Culture media were obtained from Invitrogen Life Technologies. All other chemicals were from Sigma.

Cell culture. HRP-1 cells were maintained in RPMI 1640 culture medium, pH 7.4, containing 1 mM sodium pyruvate, 50 μM β -mercaptoethanol, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 10% heat-inactivated fetal bovine serum. The cells were seeded in 75-cm 2 flasks and incubated at 37°C with 5% CO_2 .

Transport measurements. Transport was measured in HRP-1 cell monolayers cultured in transwell chambers (Costar, Cambridge, MA). To prepare cell monolayers, cells were seeded at a density of 1.2×10^5 cells per polycarbonate membrane (0.4- μm pore size, collagen coated) in transwell cell chambers, which were placed in 12-well cluster plates. The volumes of medium inside and outside the chambers were 0.5 and 1.5 ml, respectively. Fresh medium was replaced every 2 days, and the cells were used between the 3rd and 4th days after seeding. Transport was measured in Dulbecco's phosphate-buffered saline (PBS), containing 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4 , 1 mM CaCl_2 , and 0.5 mM MgCl_2 supplemented with 5 mM D-glucose. To measure the cellular uptake of radiolabeled substrates, the reaction was initiated by adding each buffer-containing substrate to the apical side of the monolayers. After incubation for a specified period, the uptake medium was aspirated and discarded, and the membrane was rapidly washed three times with ice-cold PBS. The cell monolayers on the membrane were solubilized in 0.2 N NaOH, neutralized in 0.2 N HCl, and aliquoted for liquid scintillation counting. The protein concentration was determined using the Bradford dye-binding procedure (3). For the inhibition studies, uptake of radiolabeled substrates was measured in the presence of unlabeled compounds as indicated.

Data analysis and statistics. Kinetic uptake parameters such as the concentration at half-maximal transport velocity (K_m , Michaelis-Menten constant), maximum uptake velocity (V_{max}), and passive membrane permeability coefficients (P_{diff}) were calculated using SigmaPlot software (SPSS, Chicago, IL) by nonlinear least squares regression analysis. Calculations utilized the following equation, where V is the total flux and $[S]$ is the estrone sulfate concentration.

$$V = \frac{V_{\text{max}}[S]}{K_m + [S]} + P_{\text{diff}}[S]$$

Statistics. To test the significance of differences between data sets, Student's t -test was performed.

RESULTS

Kinetics of [^3H]estrone sulfate transport. At confluence, HRP-1 cells form an epithelium with separate apical and basolateral membrane domains containing different complements of membrane proteins. Initial

experiments were performed by measuring the apical uptake into HRP-1 cells of [^3H]estrone sulfate, a common substrate for members of the OATP family. As shown in Fig. 1, the apical uptake of [^3H]estrone sulfate increased linearly for ~ 5 min and reached a steady state between 5 and 10 min. Therefore, an uptake period of 3 min was chosen for future studies. The initial rate for the uptake of estrone sulfate over a wide range of estrone sulfate concentrations (50 nM-10 μM) was then determined (Fig. 2). Kinetic analysis revealed a saturable uptake mechanism, which was superimposed by a nonsaturable component, presumably simple diffusion. Fitting the data to Eq. 1 yielded a K_m of 4.67 μM , a V_{max} of 1.13 $\text{pmol} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$, and a P_{diff} of 0.044 $\mu\text{l} \cdot \text{mg}^{-1} \cdot \text{min}^{-1}$.

Temperature dependence. The uptake of estrone sulfate in HRP-1 cells was further characterized at a decreased temperature (Fig. 3). Incubation at 4°C significantly reduced the rate of uptake compared with that seen at room temperature, indicating that the transport of estrone sulfate is a temperature-dependent process.

Na^+ dependence. The role of Na^+ in estrone sulfate uptake by HRP-1 cells was investigated in this study (Fig. 4). This was done by examining the effect of isosmotically replacing Na^+ (137 mM) in the incubation buffer with the monovalent cation choline (137 mM choline chloride) or Li^+ (137 mM LiCl). The results showed that choline ion substitution for Na^+ led to a significant decrease in estrone sulfate uptake compared with that in cells incubated in Na^+ buffer. Replacement of Na^+ by Li^+ completely restored the stimulatory effect by Na^+ , demonstrating that the transport function is Na^+ dependent and tolerates replacement of Na^+ by Li^+ .

The role of Na^+ in estrone sulfate uptake was further examined using ouabain, a specific Na^+/K^+ -ATPase inhibitor. Preincubation of HRP-1 cells with 1 mM ouabain significantly inhibited cell uptake of estrone

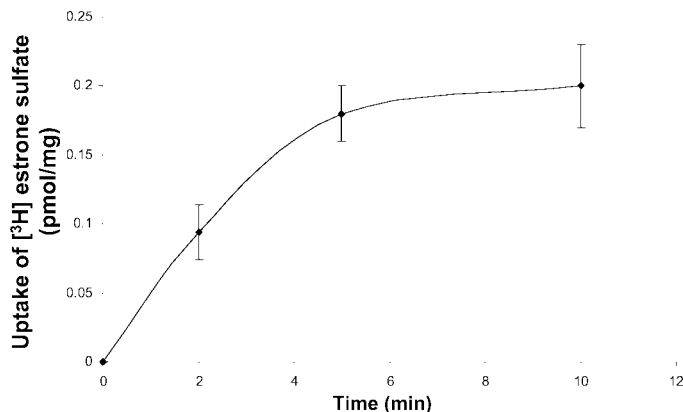


Fig. 1. Time course of [^3H]estrone sulfate uptake in HRP-1 cells. Cell monolayers were grown on collagen-coated transwells. [^3H]estrone sulfate (50 nM) was added to the apical chambers of the transwells. At specified time points, cells were washed twice with ice-cold PBS, lysed with 0.2 N NaOH, neutralized with 0.2 N HCl, and measured for radioactivity. Each value represents the mean \pm SD of 3 experiments.

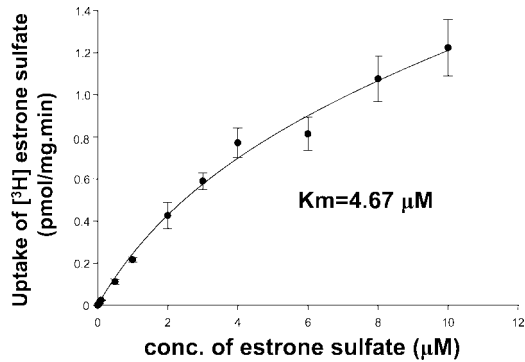


Fig. 2. Concentration dependence of estrone sulfate uptake in HRP-1 cells. Cell monolayers were grown on collagen-coated transwells. [³H]estrone sulfate (0.05–10 µM) was added to the apical chambers of the transwells and incubated for 3 min. Cells were then washed twice with ice-cold PBS, lysed with 0.2 N NaOH, neutralized with 0.2 N HCl, and measured for radioactivity. Each value represents the mean ± SD of 3 experiments. Curve represents the calculated fit of the data to Eq. 1 as described in MATERIALS AND METHODS.

sulfate (Fig. 5), suggesting that the transport is driven by the (transmembrane) Na⁺ gradient established by Na⁺-K⁺-ATPase.

pH dependence. In a separate experiment we examined the effect of varying the incubation buffer pH over the range of 5.0 to 8.5 (Fig. 6). The results showed an ~65–100% increase in estrone sulfate uptake at an acidic pH of 5.0 compared with uptake observed at pH 7.4 and pH 8.5. To test whether the uptake process is driven by an inwardly directed proton gradient, cells were pretreated separately with amiloride, carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP), and nigericin (Fig. 7). Amiloride is a specific Na⁺/H⁺ exchanger. FCCP, a protonophore, is an uncoupler of oxidative phosphorylation that equilibrates proton concentration across biological membranes. Nigericin is a K⁺/H⁺ ionophore that mediates the electroneutral exchange of a proton for a potassium ion across biological membranes, leading to a decrease in membrane pH gradient without affecting the membrane potential. None of these treatments blocked the increase of the uptake in the presence of an inwardly directed H⁺

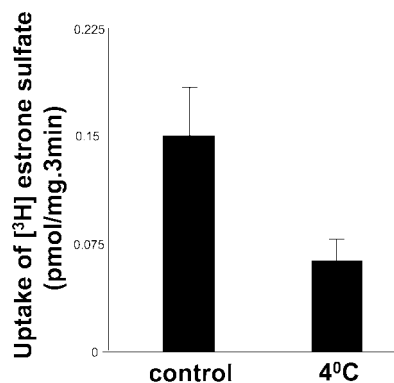


Fig. 3. Effect of decreased temperature on apical uptake of estrone sulfate. Apical uptake of [³H]estrone sulfate (50 nM) after a 3-min incubation was measured at both room temperature and 4°C. Each value represents the mean ± SD of 3 experiments ($P < 0.01$).

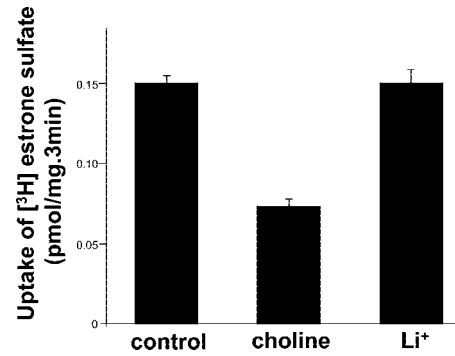


Fig. 4. Effect of extracellular Na⁺ on uptake of estrone sulfate. Apical uptake of [³H]estrone sulfate (50 nM) after 3-min incubation was measured in the presence or absence of Na⁺ (Na⁺ was replaced by choline or Li⁺). Each value represents the mean ± SD of 3 experiments ($P < 0.01$).

gradient (incubation medium pH 5.0), ruling out a H⁺ cotransport process.

Substrate specificity. We first examined the substrate selectivity of the transport system by competition studies (Fig. 8A). *cis*-Inhibitory effects of [³H]estrone sulfate were observed for unlabeled steroid sulfates, including estrone sulfate, β-estradiol-3,17-disulfate, 17β-estradiol-3-sulfate, and DHEAS. The transport was unaffected by *p*-aminohippurate (PAH), taurocholate, and tetraethylammonium.

We then further evaluated several radiolabeled compounds in terms of whether they were taken up into the cells via the same transport system. As shown in Fig. 8B, the uptake of [³H]DHEAS was *cis*-inhibited by unlabeled DHEAS and estrone sulfate. Vice versa, the uptake of [³H]estrone sulfate was *cis*-inhibited by unlabeled estrone sulfate and DHEAS (Fig. 8A). This mutual inhibition indicated that both estrone sulfate and DHEAS are transported into the cells by the same transport system. In contrast, the uptake of [¹⁴C]taurocholate was not inhibited by unlabeled estrone sulfate and DHEAS (Fig. 8B), suggesting that taurocholate and estrone sulfate do not share the same transport pathway.

Regulation. After identification of the existence of a carrier-mediated system for organic anion uptake by

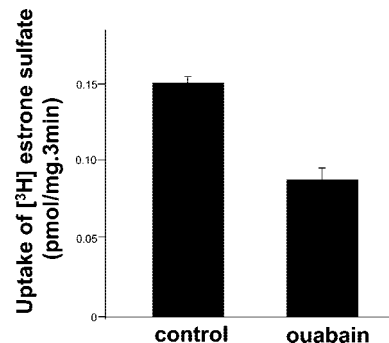


Fig. 5. Effect of ouabain on uptake of estrone sulfate. Cells were incubated with 1 mM ouabain for 1.5 h, and then uptake of [³H]estrone sulfate (50 nM) was measured. Each value represents the mean ± SD of 3 experiments ($P < 0.01$).

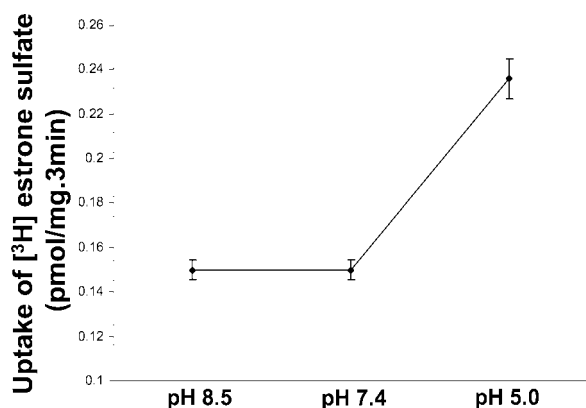


Fig. 6. Effect of incubation buffer pH on uptake of estrone sulfate. Uptake solutions were adjusted to pH 5.0, 7.4, and 8.5, and 3-min apical uptake of [³H]estrone sulfate (50 nM) was then measured. Each value represents the mean \pm SD of 3 experiments ($P < 0.01$).

HRP-1 cells and the characterization of its nature, we examined the possible regulation of the function of this carrier (Fig. 9). Forskolin activates adenylyl cyclase, and treatment with this drug has been shown to down-regulate riboflavin uptake in placental BeWo cells (13). 8-Bromo-cAMP (8-Br-cAMP), a membrane-permeable cAMP analog, has been demonstrated to stimulate the expression of the placental facilitative glucose transporter-1 (23). Incubation of HRP-1 cells with 100 μ M forskolin or 250 μ M 8-Br-cAMP for 3 h resulted in a significant increase in [³H]estrone sulfate uptake. This increased uptake was further enhanced in the presence of 3-isobutyl-1-methylxanthine, which prevents degradation of cAMP by inhibiting cyclic nucleotide phosphodiesterase.

DISCUSSION

During pregnancy, the placenta provides a barrier separating the maternal and fetal compartments. Transporters differentially expressed in the maternal-facing apical membrane and fetal-facing basolateral membrane of the placenta perform the critical task of supplying nutrition from the mother to the developing fetus and eliminating wastes from the fetus. However, many xenobiotics, including therapeutic agents, environmental pollutants, and toxins, bear structural similarity to physiological substrates and have the potential to be recognized by the transporters. Therefore a full understanding of the transport process both in the apical (maternal-facing) and basolateral (fetal-facing) membranes of the placenta and the transporters involved is of clinical, pharmacological, and therapeutic importance.

The present study reports the existence of an organic anion transport system with high affinity for estrone sulfate and DHEAS in the apical membrane of HRP-1 cells. Supporting evidence for the presence of such a transport system include 1) saturable uptake kinetics (Fig. 2), 2) significant temperature dependence for the uptake of estrone sulfate (Fig. 3), and 3) inhibition of uptake in the presence of structural analogs (Fig. 8).

The transport function is Na⁺ dependent and Li⁺ tolerant (Fig. 4). Replacement of Na⁺ by choline led to a significant decrease in estrone sulfate uptake. In contrast, replacement of Na⁺ by Li⁺ completely restored the stimulatory effect by Na⁺. Na⁺-dependent and Li⁺-tolerant transport has been reported for transporters such as N-system amino acid transporters (5) and phosphate transporters (29). However, to our knowledge, such a transport system has not been described for organic anion transporters.

Na⁺-K⁺-ATPase was previously reported to be present in human placenta (28). Our result (Fig. 5) showed that treatment of cells with ouabain, a specific inhibitor of Na⁺-K⁺-ATPase, inhibited estrone sulfate uptake, suggesting that a Na⁺ gradient, established by Na⁺-K⁺-ATPase, is required for the transport function.

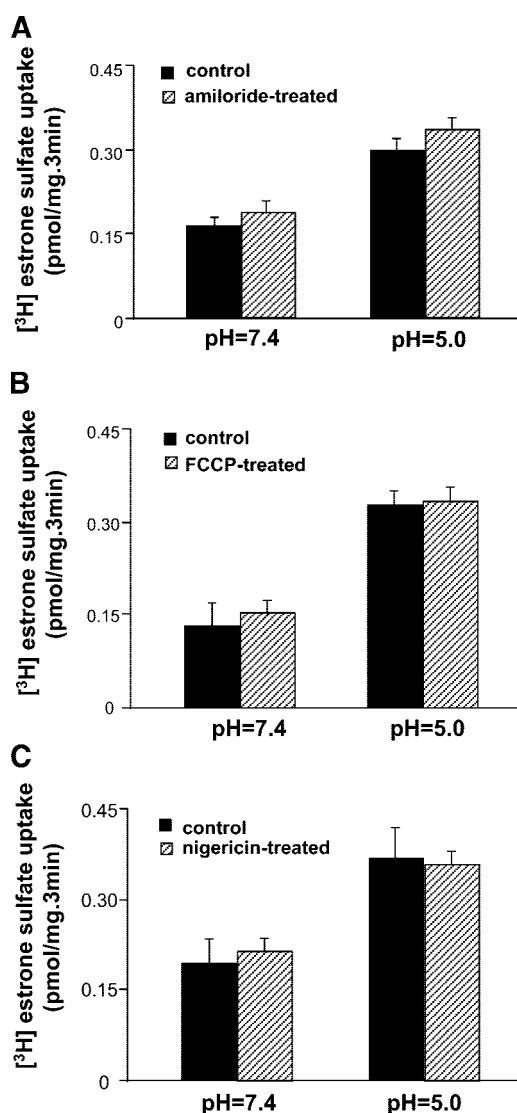


Fig. 7. Effects of proton gradient on uptake of estrone sulfate. Cells were preincubated for 1 h with 1 mM amiloride (A), 50 μ M carbonyl cyanide 4-trifluoromethoxyphenylhydrazone (FCCP; B), and 2.5 μ M nigericin (C), and uptake of [³H]estrone sulfate (50 nM) was measured at pH 7.4 and 5.0. Each value represents the mean \pm SD of 3 experiments ($P < 0.01$).

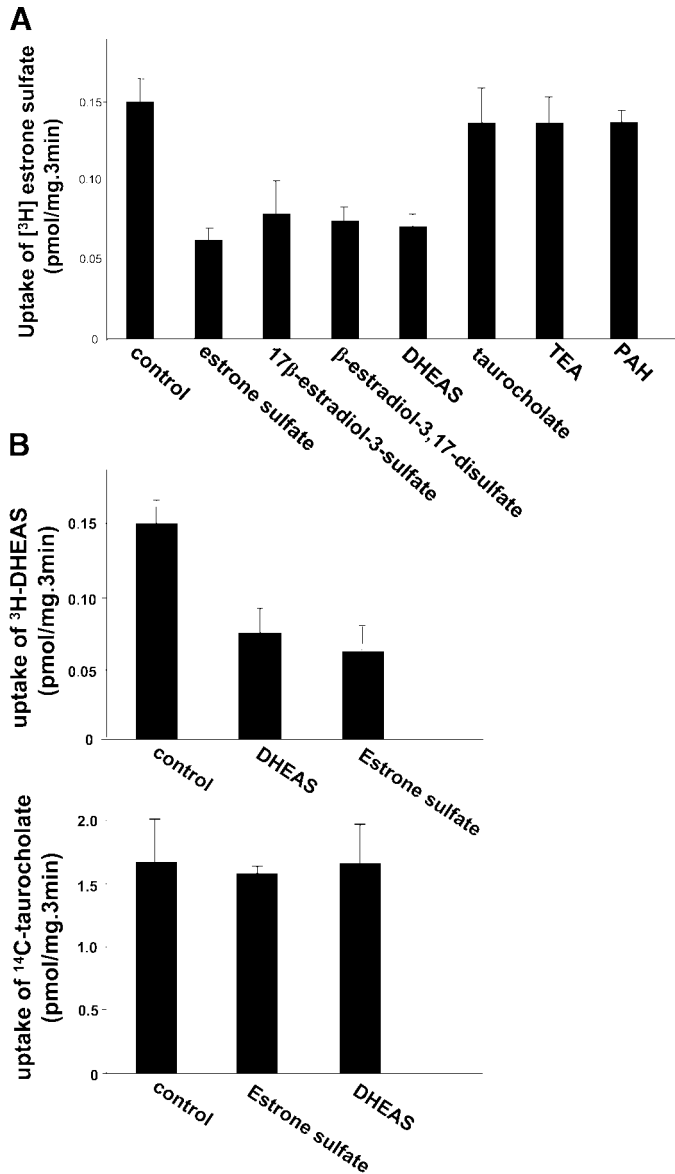


Fig. 8. Substrate specificity. A: inhibition of estrone sulfate uptake by unlabeled organic anions. DHEAS, dehydroepiandrosterone 3-sulfate; TEA, tetraethylammonium; PAH, *p*-aminohippurate. Uptake of 50 nM [³H]estrone sulfate was measured in the presence of 50 μ M unlabeled organic anions. B, top: uptake of [³H]DHEAS (50 nM) in the presence of 50 μ M unlabeled estrone sulfate or DHEAS; bottom: uptake of [¹⁴C]taurocholate (1 μ M) in the presence of 1 mM unlabeled estrone sulfate or DHEAS. Each value represents the mean \pm SD of 3 experiments ($P < 0.01$).

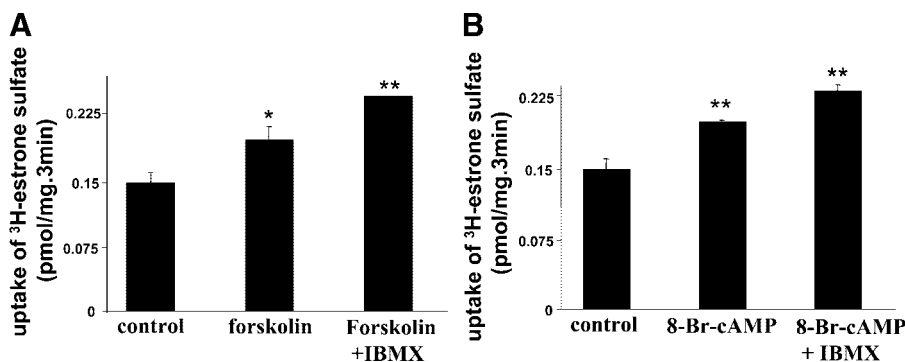


Fig. 9. Effect of forskolin and 8-bromo-cAMP (8-Br-cAMP) on estrone sulfate uptake. Cell monolayers were preincubated for 3 h with 100 μ M forskolin (A) or 250 μ M 8-Br-cAMP (B) with or without 1 mM IBMX, and then apical uptake of [³H]estrone sulfate (50 nM) was determined. Each value represents the mean \pm SD of 3 experiments (* $P < 0.05$ and ** $P < 0.01$ vs. control).

We showed that increasing the H⁺ concentration in the incubation medium by lowering the incubation buffer pH led to a marked increase in estrone sulfate uptake (Fig. 6). Several possible mechanisms could explain the observed phenomenon. For example, the effect of pH on estrone sulfate uptake may represent the existence of an estrone sulfate/H⁺ cotransport mechanism. A Na⁺/H⁺ exchanger is known to be present in the placenta membrane (7), which maintains an inwardly directed H⁺ gradient. To clarify this assumption, we examined the effect of amiloride, a specific inhibitor of Na⁺/H⁺ exchanger, on estrone sulfate accumulation. As clearly shown in Fig. 7A, pretreatment of cells with amiloride failed to block the stimulation of estrone sulfate uptake caused by lowering the medium pH. Furthermore, pretreatment of cells with FCCP (Fig. 7B) and nigericin (Fig. 7C), two reagents that disrupt the H⁺ gradient across the membrane, was unable to block the increase of the estrone sulfate uptake in the presence of an H⁺ gradient. These results suggest that proton transport is not directly involved in estrone sulfate uptake. It is then possible that the stimulation of estrone sulfate uptake at reduced pH (pH 5.0) may arise from a higher rate of nonionic diffusion due to a lower degree of dissociation of estrone sulfate. However, estrone sulfate is a sulfuric acid monoester. The acidic dissociation constant for such a compound is <0 . It can be calculated that, at pH 5.0, almost all of the estrone sulfate is in its dissociated form. Hence, it seems unlikely that the stimulation of estrone sulfate uptake can be attributed to an increased diffusion of an undissociated/un-ionized form. Therefore, the increase in estrone sulfate uptake at a reduced pH might be explained by a direct effect of pH on the estrone sulfate uptake carrier.

Our studies on substrate specificity using radiolabeled compounds (Fig. 8, A and B) demonstrated that the estrone sulfate and DHEAS are transported into the cells by this system. In humans, DHEAS represents the major circulating steroids secreted by the adrenal cortex. DHEAS not only serves as a precursor for endogenous estrogen synthesis, which is important for continuation of a pregnancy, but also exerts significant neuropsychiatric effects (21, 33). DHEAS induces an expressed feeling of remarkable psychological and physical well-being, notably with improved quality of sleep, greater energy, and an increased ability to han-

dle stress. DHEAS at high blood concentrations, however, shows undesirable effects on the fetus (e.g., intrauterine growth retardation) (27). Estrone sulfate and DHEAS are common substrates for a group of OATPs. They are also the substrates for OAT3 and OAT4, two members of the OAT family. Leazer and Klaassen (19) recently showed with an RT-PCR approach that mRNAs for these multispecific OATs are expressed at various abundances in rat placenta, although whether or not any of these genes produces a functional transport protein in this organ is not known. However, the functional characteristics of the transport system described in the present studies seem to rule out the possibility of the involvement of these transporters for the following reasons. First, these OATs mediate Na^+ -independent transport of their substrates. We showed that the transport of estrone sulfate was Na^+ dependent. Second, taurocholate, a common substrate for members of the OATP family (11), and PAH, a substrate for OAT3 (34), failed to inhibit the estrone transport (Fig. 8A). Furthermore, the uptake of radiolabeled taurocholate was not inhibited by unlabeled estrone sulfate and DHEA (Fig. 8B), suggesting that taurocholate and estrone sulfate do not share the same transport system described in the present study. OATP-B and OAT4 have recently been localized to the basolateral membrane of human placenta (31), which is different from the apical transport activity observed in our study. The rat isoform of OATP-B is Oatp9. It would be interesting to explore whether Oatp9 or rat OAT4 (not yet cloned) is involved in the basolateral uptake/exit of organic anions in HRP-1 cells. Therefore, the transport system characterized in the present study appears to be different from the known OATP and OAT transporter families described above. The identity of the rat placental cell OAT(s) needs to be investigated further.

In this study, we also provided evidence that intracellular cAMP stimulated transport activity. There are several possible mechanisms that could contribute to the stimulation of the transport activity. For example, it could result from an insertion of transporter proteins (performed in the intracellular vesicles) into the plasma membranes. Many receptors, transporters, and channels are regulated by this mechanism in response to stimuli (4, 8, 15, 18, 25). The stimulation of transport activity could also result from an increased affinity of the transporter for its substrates due to the conformation change of the transporter, which could be brought about through its phosphorylation by cAMP-dependent protein kinase pathways. Further studies are needed to differentiate between these possibilities.

In summary, we identified an organic anion transport system on the apical membrane of HRP-1 placental cells, which seems to be different from the previously described organic anion transporters from OATP and OAT families. This transport system appears to be regulated by cellular cAMP levels.

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DISCLOSURES

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