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Modification of OATP2B1 mediated transport by steroid hormones*

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Abstract

The family of the organic anion transporting polypeptides forms an increasing group of uptake transport proteins with a wide substrate spectrum. While the expression of some members of this group like OATP-A or C is limited to special tissues like liver or brain, the organic anion transporting polypeptide 2B1 (OATP-B / SLCO2B1) is expressed in many organs including liver, placenta, mammary gland, brain, and intestine. However, little is known about its function in those tissues, because only a limited number of compounds like dehydroepiandrosterone-sulfate (DHEAS) and estrone-3-sulfate (E3S) have been characterized as OATP2B1 substrates.

To further elucidate the role of OATP2B1 on steroid transport, we examined the influence of steroid hormones on OATP2B1-mediated E3S and DHEAS uptake using OATP2B1-overexpressing MDCKII cells. We could identify unconjugated androgens (e.g. testosterone) as potent inhibitors for OATP2B1. In contrast, gestagenes like progesterone enhanced E3S uptake in a concentration dependent manner to up to 300 % of the control, accompanied by a significant decrease in OATP2B1 K_m value for E3S (control: K_m = 14 µM; in the presence of 31.6 µM progesterone K_m = 3.6 µM). Moreover, we could demonstrate that testosterone and progesterone are not substrates of OATP2B1, indicating an allosteric mechanism for the observed effects. Furthermore, we could show that progesterone enhances the OATP2B1-dependent pregnenolone sulfate transport.

Taken together, the results indicate functional modification of OATP2B1-mediated E3S and DHEAS as well as pregnenolone sulfate transport through steroid hormones like progesterone. These effects can have physiological consequences for the organ-specific uptake of steroids.

Introduction

Organic anionic polypeptide transporters (OATPs) belong to the solute carrier (SLC 21; new classification SLCO (Hagenbuch and Meier, 2004)) family, a group of multispecific uptake proteins which mediate the sodium-independent transport of a wide variety of endogenous and xenobiotic compounds, which are in general large amphiphatic molecules and are mainly bound to albumin under physiological conditions. So far, 11 members of this family have been identified in humans: OATP1A2 (OATP-A), OATP2B1 (OATP-B), OATP1B1 (OATP-C), OATP3A1 (OATP-D), OATP4A1 (OATP-E), OATP1C1 (OATP-F), OATP4C1 (OATP-H), OATP5A1 (OATP-J), OATP6A1 (OATP-I), OATP1B3 (OATP8) and OATP2A1 (PGT) (Hagenbuch and Meier, 2004). These transporters exhibit homology among each other with an overall amino acid identity of 31%-82%. They demonstrate a high variability in tissue distribution as well as in functional characteristics (reviewed in (Hagenbuch and Meier, 2003;Hagenbuch and Meier, 2004)). OATP1B1 for example has a broad substrate specificity, which comprises bile salts, prostaglandins and steroid conjugates, but is exclusively localized in liver, where its main function might be the elimination of compounds (Cui et al., 2001;Tamai et al., 2000;Kullak-Ublick et al., 2001). On the other hand, OATP2B1 has, as known so far, a narrow substrate spectrum, which includes estrone-3-sulfate (E3S), dehydroepiandrosterone-sulfate (DHEAS) and bromosulfophthalein, but shows a broad tissue distribution (Tamai et al., 2000;Pizzagalli et al., 2003). OATP2B1 expression could be demonstrated in many organs as liver, placenta, mammary gland, brain, and small intestine (Kullak-Ublick et al., 2001). In the latter, an apical expression in epithelial cells and a pH-sensitive transport with higher activity at acidic pH (transport of pravastatin) was observed (Kobayashi et al., 2003). Satoh et al. (Satoh et al., 2005) however, could not demonstrate pH-sensitivity of the E3S transport, which might by due to different cell types used. Interestingly, despite its apical localization in the gastrointestinal tract OATP2B1 shows a basal/basolateral expression in other tissues. For example, OATP2B1 has been identified as a carrier in human

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mammary gland with localization in myoepithelial tissue, surrounding ductal epithelial cells and in the basal membrane of the human placental syncytiotrophoblast, where it might be relevant for supply of steroid hormone precursors like DHEAS to the fetoplacental unit (Pizzagalli et al., 2003;St Pierre et al., 2002;Ugele et al., 2003). DHEAS, the transport form of DHEA, represents the major circulating steroid in human blood, reaching concentrations up to 10 µM (Baulieu, 1996). It is secreted predominantly by the adrenal cortex and serves as the principal conjugated prohormone for the biosynthesis of both estrogenic and androgenic steroids in peripheral tissues. It acts, in addition, as a neuroactive steroid in brain, where it can also be synthesized (Baulieu and Robel, 1998). DHEAS has been identified as substrate for several members of the OATP family, in particular OATP1A2, which is mainly expressed in brain (Kullak-Ublick et al., 1998), the liver-specific OATP1B1 (Konig et al., 2000a) and OATP1B3 (Konig et al., 2000b) as well as for OATP2B1 with its wide tissue distribution (Kullak-Ublick et al., 2001).

To investigate the role of OATP2B1 in transport and supply of steroid hormone metabolites and precursors, we studied the effects of steroid hormones on OATP2B1 transport activity with regard to E3S and DHEAS uptake. In this paper we describe for the first time a stimulatory effect of progesterone on E3S and DHEAS uptake and characterize its kinetic parameters. Moreover, we identify pregnenolone sulfate as a new substrate for OATP2B1.

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Materials and Methods

Cell Culture and Substances

Madin-darby-canine kidney (MDCKII) cells were grown in DMEM medium containing 10 % fetal calf serum, 2 mM L-glutamine, 1 % MEM nonessential amino acids and penicillin/streptomycin (0.5 U/ml and 150 µg/ml). Cells were incubated at 37 °C in an atmosphere containing 5% CO_2. Cells were incubated with 2.5 mM sodium butyrate 24 h prior to transport experiments to enhance the expression of the recombinant protein.

Radiolabeled substances were obtained as indicated in the respective section. All other substances used for transport studies were obtained from Sigma-Aldrich (Sigma-Aldrich Chemie GmbH, Munich, Germany).

Cloning and Transfection

The full length OATP2B1 cDNA was amplified using reverse transcribed RNA from human placenta (forward primer: 5´- agctcactgcactccagcagtcatgg -3´; reverse primer: 5´- aaaggactcagaggaggtactgctgtggctgc -3´) and cloned into the mammalian expression vector pcDNA3.1/hygro (Invitrogen, Carlsbad, CA, USA). After verifying the sequence by cycle sequencing and matching against the reference sequence (accession number: AB026256.1) MDCKII cells were transfected with this construct using the *FuGENE 6 Transfection Reagent* (Roche, Penzberg, Germany). Cells were selected for antibiotic resistance using 0.6 mg/ml hygromycin B (Invitrogen).

Immunofluorescence Microscopy

Protein localization was investigated by confocal laser scanning immunofluorescence microscopy. For OATP2B1 a polyclonal antibody described before was used (rabbit, dilution 1:200)(Grube et al., 2005). After washing with PBS the cover slides were incubated for 1 h

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with Alexa Fluor® 488 labeled IgG (anti-rabbit IgG). Staining of nuclei was performed using a 1:2000 dilution of TOTO®-3-iodide in DAKO® Fluorescent Mounting Medium (DakoCytomation, Carpinteria, CA, USA). The secondary antibodies as well as the TOTO® dye were purchased from Molecular Probes (Invitrogen, Carlsbad, CA, USA).

Immunoblot analysis

Crude membrane fractions were loaded onto a 7.5 % sodium dodecylsulfate-polyacrylamid gel after incubation in sample buffer at 95 °C for 10 min. Immunoblotting was performed using a tank blotting system (BioRad, Hercules, CA, USA) and an enhanced chemiluminescence detection system (Amersham Pharmacia Biotech, Freiburg, Germany). The OATP2B1 primary antibody was diluted in TBS containing 0.05 % Tween 20 and 5 % bovine serum albumin. A secondary horse-radish peroxidase-conjugated goat anti-rabbit antibody (BioRad, Hercules, CA, USA) was used at a 1:2000 dilution.

Transport studies

For characterization of OATP2B1 transfection the respective cells were incubated with [^{3}H]estrone-3-sulfate (E3S) (specific activity: 50 Ci/mmol) and [^{3}H]dehydroepiandrosterone -3-sulfate (DHEAS) (specific activity: 60 Ci/mmol; both from Hartmann Analytic, Braunschweig, Germany) for 5 or 10 min, respectively; washed four times with ice-cold PBS and lysed with 0.2 % SDS. An aliquot was mixed with 4 ml scintillation cocktail (Rotiszint, Roth, Karlsruhe, Germany) and measured in a scintillation beta-counter (type 1409, LKB-Wallac, Turku, Finland). For E3S uptake inhibition experiments as well as for transport studies with [^{3}H]testosterone, [^{3}H]progesterone and [^{3}H]pregnenolone sulfate (all 50 Ci/mmol; Biotrend, Köln, Germany) cells were seeded out in 24 well dishes, cultured to confluence and incubated for indicated times with the respective substance at 37°C. Rates of transport are given in mol x mg protein^{-1} x min^{-1} or as ratio of control.

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All transport studies were performed using an incubation buffer containing 140 mM $NaCl_2$, 5 mM KCl, 1 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.5 mM $CaCl_2$, 5 mM glucose and 12.5 mM Hepes (pH 7.3).

Statistical methods

Values are represented as means ± SD. Graphs and calculation were in general prepared using Microsoft excel or GraphPad Prism software 3.0 (GraphPad Software, San Diego, CA, USA) software. Students't-test was performed to determine statistical significance. Differences were considered significant at $p < 0.05$. The EC_{50} values were calculated by nonlinear regression from a sigmoidal dose-response curve (variable slope, bottom value ≥ 0) using GraphPad. K_m and V_{max} values were also calculated by nonlinear regression using the equation: $Y = (V_{max} * X) / (Km + X)$ by Graph-Pad.

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Results:

Characterization of the OATP2B1-overexpressing cells

Characterization was performed by Western blot and immunofluorescence technique. Therefore, OATP2B1-transfected MDCKII cells and non-transfected cells were cultured until confluence and treated with 2.5 mM sodium-butyrate for 24 h. For Western blotting, cells were harvested and crude membrane fractions were obtained. After gel electrophoresis proteins were blotted onto a nitrocellulose membrane and OATP2B1 expression was detected by antibody staining. The OATP2B1-transfected cells exhibit a strong band at approximately 84 kDa, which corresponds to the published molecular mass of OATP2B1 (St Pierre et al., 2002), compared to non-transfected MDCKII cells. To verify this result and to determine the localization of OATP2B1 immunofluorescence staining was performed. The OATP2B1 expression was restricted to the plasma-membrane of the transfected cells (data not shown).

E3S and DHEAS uptake studies

As a first screening approach, OATP2B1-transfected cells were incubated with tritium-labeled E3S and DHEAS in the presence of various steroids. Substances like estrone, estriol, β-estradiol, mifepristone, testosterone and pregnenolone sulfate significantly inhibit E3S uptake. In contrast, steroids like hydroxyprogesterone, pregnenonlone and progesterone enhance the E3S uptake depending on their concentration (Tab. 1). For the uptake of DHEAS similar results could be observed, however, the inhibitory effects were less intense, while the inducing effects were much higher compared to the E3S results (Tab. 2). The inhibition/stimulation the compounds exerted on E3S uptake were similar to the effects on DHEAS transport. Interestingly, divergent results were obtained for estrone: E3S uptake was inhibited (68 % of control for 100 µM E3S) while transport of DHEAS was induced up to 280 % of control by estrone concentration of 10 and 100 µM (288 % and 283 %).

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To further characterize the inhibitory effects of testosterone and mifepristone as well as the stimulatory effect of progesterone on E3S and DHEAS uptake mediated by OATP2B1 the concentration of the half-maximal effect (EC_{50}) was determined. For the inhibition of testosterone EC_{50} concentrations of 21.2 ± 5.1 µM for E3S and 15.4 ± 1.3 µM for DHEAS uptake were calculated, for mifepristone the respective concentration were 4.7 ± 0.7 µM for E3S and 2.2 ± 0.8 µM for DHEAS transport. The observed maximal inhibition of E3S and DHEAS uptake by testosterone and mifepristone was about 75 % of control for both substances. In contrast, progesterone induces the uptake of E3S to a maximum of 200 % and for DHEAS to 400 % of control. These stimulatory effects show half maximal concentrations of 1.5 ± 0.6 µM for E3S and 4.9 ± 1.5 µM for DHEAS (EC_{50} values are given as mean ± SD for 3 independent experiments). The maximal effects were obtained for a progesterone concentrations between 10 and 30 µM, while higher concentrations lead to decreased induction (Fig. 1).

Transport of labeled progesterone and testosterone

To determine if the interaction of testosterone and progesterone with the OATP2B1-mediated uptake of E3S and DHEAS is caused by a direct competition, OATP2B1-mediated transport of these compounds was tested. Therefore, transfected and non-transfected cells were incubated with tritium-labeled progesterone, testosterone, E3S and DHEAS. As shown in table 3, E3S and DHEAS were taken up in a significantly higher amount into transfected as compared to control cells (11.1 ± 1.1 fmol/mg/min to 301 ± 33 fmol/mg/min for E3S and 11.4 ± 1.9 fmol/mg/min to 36.3 ± 3.5 fmol/mg/min for DHEAS). However, no significant difference in the uptake of testosterone could be observed between OATP2B1-transfected and control cells. Moreover, for progesterone an enhanced uptake into non-transfected cells was observed. Next, we tested whether E3S or DHEAS stimulated the uptake of tritium-labeled progesterone. While progesterone had a stimulatory action upon E3S and DHEAS uptake,

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such reciprocal action was missing for progesterone uptake by E3S or DHEAS (data not shown).

Further experiments were carried out to investigate the stimulatory effect of progesterone on OATP2B1-mediated DHEAS uptake. OATP2B1-transfected cells were incubated with [^{3}H] DHEAS and different progesterone concentrations in the presence of DMSO (0.1 %), 1 µM and 10 µM mifepristone (Fig.2). The calculation of the EC_{50} revealed values of 2.8 ± 1.5 µM for the DMSO control, 5.3 ± 2.9 µM for 1µM mifepristone and 2.4 ± 0.4 µM for 10 µM mifepristone (EC_{50} values are given as mean ± SD for 3 independent experiments). However, the maximal induction effect decreases form 500 % (control) to 144 % in the presence of 10 µM mifepristone, indicating a non competitive interaction of progesterone and mifepristone.

Kinetic studies

Additional experiments were carried out to analyze the interaction type of E3S transport and progesterone. Transfected cells were incubated with different concentrations of [^{3}H] E3S (0.1 to 31.6 µM) in the presence or absence of progesterone (1 to 100 µM). Results are displayed as a Lineweaver-Burk plot (Fig. 3A) and Eadie-Hofstee plot (Fig. 3B). K_m and V_{max} values were calculated by non linear regression in dependence of the progesterone concentrations (Fig. 3C). Up to progesterone concentrations of 31.6 µM the affinity of OATP2B1 towards E3S increases 4-fold from K_m = 14 µM (DMSO control) to K_m = 3.6 µM (31.6 µM progesterone), while higher progesterone concentrations (K_m = 7.2 µM for 100 µM progesterone) resulted in a decrease, again. In contrast, the maximal velocity of E3S uptake (V_{max}) is decreasing from 169 pmol/min/mg (control) to 106 pmol/min/mg (for 100 µM progesterone).

OATP2B1 and Pregnenolone sulfate

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Pregnenolone sulfate (10 µM) significantly inhibited E3S and DHEAS transport to 28 % and 43 % of the control, respectively (Tab. 1 and 2); moreover, an EC_{50} value of 3.5 ± 1.5 µM was calculated for E3S uptake (Fig 4A). To determine, if pregnenolone sulfate is like other sulfates subject of OATP2B1-mediated transport, pregnenolone sulfate uptake experiments were performed in transfected and non-transfected cells. Incubation with pregnenolone sulfate revealed a time-dependent OATP2B1-mediated uptake. This transport could be further stimulated by addition of 10 µM progesterone, while progesterone had no effect on pregnenolone sulfate uptake into non-transfected cells (Fig.4B and C). To further characterize the stimulatory effect of progesterone on pregnenolone sulfate uptake the concentration-dependency was evaluated over a concentration range of 1.6 µM to 100 µM. While progesterone had no effect on MDCKII cells increasing concentrations of progesterone stimulated pregnenolone sulfate uptake with an EC_{50} value of 5.18 µM.

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Discussion

OATP2B1 (formerly known as OATP-B) is a member of the organic anion transporting polypeptide (OATP) family and is widely expressed in human tissues. For example, OATP2B1 expression could be demonstrated in the basolateral hepatocyte membrane, the basal membrane of the placental syncytiotrophoblast, the apical membrane of the enterocytes and in myoepithelial cells of the mammary gland (Konig et al., 2000a;St Pierre et al., 2002;Kobayashi et al., 2003;Pizzagalli et al., 2003). In contrast to other OATPs like OATP1B1 (OATP-C), which is described as a liver specific uptake system for various glucuronide- and sulfate-conjugates, the spectrum of identified substrates of OATP2B1 mainly consists of sulfate-conjugates like DHEAS and E3S (Tamai et al., 2001). Because of these transport properties and its localization in tissues like the placenta or the mammary gland, which are involved in the steroid synthesis, OATP2B1 was discussed to be responsible for the uptake of precursor molecules like DHEAS for the steroid hormone synthesis (Ugele et al., 2003). Moreover, E3S, which is the best characterized substrate of OATP2B1, seems to be not only a biologically inactive elimination product of estrogen but also serves as an inactive pool for this hormone (Miki et al., 2002;Santner et al., 1986). For our inhibition experiments we used both E3S and DHEAS in a concentration of 1 µM, which is in the range of the published K_m-values of OATP2B1 for both substances (Tamai et al., 2001;Pizzagalli et al., 2003); moreover, DHEAS concentrations in a low micromolar range are also achieved under physiological conditions (Skalba et al., 2003). Taken together, an interaction of steroid hormones with the transport activity of OATP2B1 may be important. However, little is known about the interaction of OATP2B1 with steroid hormones like gestagens, estrogens and androgens. In the present study, we therefore investigated the interaction of OATP2B1-mediated transport with these compounds.

In a first screening approach using an OATP2B1-overexpressing cell system we were able to show significant inhibition of E3S and DHEAS uptake by steroid hormones or related

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compounds like testosterone or mifepristone (Tab. 1 and 2), while other steroids like progesterone and pregnenolone demonstrated stimulatory effects on the uptake of both E3S and DHEAS. In the case of mifepristone and testosterone the inhibitory effects were concentration dependent with EC_{50} values of 4.7 µM (2.2 µM) and 21.2 µM (15.4 µM) for E3S (DHEAS), respectively. For both substrates used the highest stimulatory effect of progesterone was observed for concentrations of around 10 µM, while higher progesterone concentrations were only little inductive or even inhibitory for DHEAS and E3S uptake. The mifepristone EC_{50} values are in the range of pharmacologically relevant levels (Sarkar, 2002). In contrast, the EC_{50} values for progesterone and testosterone are very high compared to systemic concentrations (physiological progesterone concentration: 5 to 35 nmol/l in women, depending on the menstrual cycle (Claydon et al., 2006)); however, serum concentrations might not reflect local conditions. This assumption may be underlined by the observation of very high concentrations in the cord blood (mean concentration: 750 nmol/l, ranging from 240 to 1670 nmol/l) (Baik et al., 2005) and the presence of elevated progesterone levels in nipple aspiration fluid of human breast (around 300 nmol/l) (Khan et al., 2005).

While direct transport or inhibition by steroids or its metabolites has already been described for some uptake transport proteins of the OATP family, as well as ABC-efflux pumps like P-glycoprotein (P-gp) or breast cancer resistance protein (BCRP) (Konig et al., 2006;Pavek et al., 2005;Frohlich et al., 2004), observations of stimulatory steroid effects on uptake transport processes are rather rare. Pizzagalli *et al.* described stimulatory effects of prostaglandin A1 on E3S uptake mediated by OATP2B1 (Pizzagalli et al., 2003). It is also notable that the observed effects of progesterone and mifepristone on OATP2B1-mediated uptake of E3S and DHEAS are similar to their influence on the activity of the ABC transporter ABCB1 (P-glycoprotein, P-gp) (Fardel et al., 1996;Shapiro et al., 1999).

Interestingly, neither testosterone, nor progesterone is transported by OATP2B1 for themselves (Tab. 3). Therefore, we hypothesize that testosterone blocks the E3S or DHEAS

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transport in a non-competitive way. For progesterone our observation excludes the possibility that the induction of E3S and DHEAS uptake is due to a progesterone-E3S- (DHEAS-) symport, which is underlined by the finding that no reciprocal stimulatory action of E3S or DHEAS on progesterone uptake was seen.

Further kinetic studies concerning the stimulatory effect of progesterone on E3S uptake led to the observation that progesterone increases the affinity of OATP2B1 towards E3S significantly (Fig. 3). Whether this effect is based on a direct modulatory interaction of progesterone with the substrate binding site of OATP2B1 or is due to an allosteric progesterone binding site as described by Shapiro *et al.* for the interaction of progesterone and P-gp, remains to be elucidated (Shapiro et al., 1999). The inhibitory action of high progesterone concentrations suggest the presence of two binding sites: one high affinity allosteric binding site, which is responsible for the stimulatory effect and the substrate binding site or a further allosteric binding site, towards which progesterone has lower affinity and an inhibitory effect.

Moreover, we were able to show that subinhibitory concentrations of the gestagene antagonist mifepristone (like 1 µM) were able to decrease the effect of progesterone on DHEAS uptake without altering the stimulatory EC_{50} concentrations of progesterone significantly, leading to the conclusion that mifepristone and progesterone are interacting in a non-competitive way with OATP2B1 (Fig.3). If mifepristone is thereby interacting directly with the DHEAS transport or is modulating a potential allosteric progesterone binding site remains to be elucidated. However, we assume, that mifepristone has a higher affinity towards a potential allosteric progesterone binding site than progesterone. This assumption would explain our findings using low mifepristone concentrations and is supported by the fact that mifepristone acts in a similar way at the intracellular progesterone receptor.

While DHEAS and E3S are already characterized as OATP2B1 substrates, pregnenolone sulfate, which represents a precursor molecule for gestagene synthesis (Chibbar and Mitchell,

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1990), is not described as an OATP2B1-substrate. However, our and other groups demonstrate a strong inhibitory effect on E3S and DHEAS uptake (St Pierre et al., 2002). Moreover, in contrast to a previous study (Pizzagalli et al., 2003) we observed a small but significant OATP2B1 dependent pregnenolone sulfate transport. This transport was enhanced by progesterone with an EC_{50} concentration for progesterone comparable to the above mentioned values.

The presented findings are especially of interest with regard to the OATP2B1 expression in hormonally regulated tissues like placenta or mammary gland (St Pierre et al., 2002;Pizzagalli et al., 2003). The placenta representing the main source of estrogens and progesterone during pregnancy is unable to convert cholesterol into estrogen due to a lack of CYP17 and is therefore highly dependent on uptake of C-19 precursor steroids like DHEAS (Voutilainen and Miller, 1986). On the other hand, the placenta or in more detail the syncytiotrophoblast is producing high levels of progesterone, which is increasing with the gestation age (Strauss, III et al., 1996;Kallen, 2004). In this context the placental progesterone might be an important factor in regulation of DHEAS and E3S uptake. Moreover, our results also indicate a progesterone enhanced uptake of pregnenolone sulfate via OATP2B1. This pathway represents a possible mechanism by which progesterone mediates the uptake of its own precursor molecules into the syncytiotrophoblast.

With respect to the mammary gland, circulating hormones (like estrone-3-sulfate) and local biosynthesis within epithelial cells and stromal fibroblasts from precursors like DHEAS and androstendione serve as estrogen-supplying mechanisms (Labrie et al., 2001). The latter mechanism becomes increasingly important during aging, since estrogen synthesis by the ovaries ceases completely during menopause (Labrie et al., 2003). As mentioned above for the placenta the mammary gland is therefore also dependent on DHEAS and E3S uptake and again OATP2B1 has been identified as the major uptake carrier. In contrast to the placenta, where the protein is localized to the basal membrane of the syncytiotrophoblast, here the

localization is restricted to the myoepithelium, where it might play a role in the supply of desulfated hormones to the adjacent ductal epithelial cells (St Pierre et al., 2002;Pizzagalli et al., 2003). Besides this general importance of OATP2B1 for the steroid metabolism in mammary gland, the stimulatory effect of progesterone on this transporter can be of relevance during pregnancy, when epithelial cells proliferate and milk-filled alveolar lobules develop in the mammary gland (Anderson and Clarke, 2004;Soyal et al., 2002)

Taken together our data demonstrate strong interactions of OATP2B1-mediated uptake of E3S and DHEAS and steroid hormones. In the case of testosterone transport of both substances was inhibited, while progesterone is stimulating E3S and DHEAS uptake. These findings are of potential relevance for uptake regulation of sulfated steroid metabolites to sex steroid responsive tissues like liver mammary gland and placenta.

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Footnotes

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Figure legends

Figure 1. Determination of the half maximal concentration of the progesterone, testosterone and mifepristone effect on OATP2B1-mediated E3S and DHEAS uptake. Cells were incubated for 10 min with [^{3}H]DHEAS (1µCi/ml; 1µM) or 5 min with [^{3}H]E3S (1µCi/ml; 1µM) in the presence of increasing concentrations of steroid hormones. Uptake is shown as % of DMSO control (mean ± SD; n = 3 for a representative experiment).

Figure 2. Interaction of progesterone and mifepristone on DHEAS uptake into OATP2B1-overexpressing cells. Cells were incubated with [^{3}H]DHEAS (1µCi/ml; 1µM) and progesterone concentrations ranging from 0 to 100 µM for 10 min at 37°C in absence (■) or presence of mifepristone (1µM (▲) or 10µM (♦), respectively) (mean ± SD; n = 3 for a representative experiment).

Figure 3. Kinetic analyses of progesterone on E3S uptake. Uptake of [^{3}H]E3S into OATP2B1 expressing cells was measured for 5 min at E3S concentrations ranging from 0.1 – 31.6 µM in the absence or presence of progesterone concentrations ranging from 1 to 100 µM. A. Lineweaver-Burk plot of the transport rates of the control (♦) or 3.16 µM (∗) and 31.6 µM (∇) progesterone incubations. B. Eadie-Hofstee plot of the same data. C. K_m (□) and V_{max}(x) values of E3S uptake plotted versus progesterone concentration. Values were calculated form the direct linear plots using GraphPad software (data represent mean ± SD, n = 3).

Figure 4. Interaction of pregnenolone sulfate and OATP2B1. A. Determination of EC_{50} value of pregnenolone sulfate on OATP2B1-mediated E3S uptake. Cells were incubated for 5 min with [^{3}H]E3S (1µCi/ml; 1µM) in the presence of increasing concentrations pregnenolone sulfate. Uptake is shown as % of DMSO control (mean ± SD; n = 3; data were obtained from three independent experiments). B. Time course of [^{3}H]pregnenolone sulfate uptake

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(0.5 µCi/ml; 1µM) into OATP2B1-transfected MDCKII (▲) and control cells (■) in the presence (closed symbol) or absence (open symbol) of 10 µM progesterone. C. Uptake of [^{3}H]-pregnenolone sulfate (0.5 µCi/ml; 1µM) into transfected (▲) and non-transfected (■) cells in the presence of different progesterone concentrations (mean ± SD (n=3)).

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Tables

Table 1. Effect of different steroids on the OATP2B1-mediated E3S uptake. OATP2B1 transfected MDCKII cells were incubated with [^{3}H] E3S (1µCi/ml; 1µM) for 5 min in the presence of indicated compounds (n = 3; *significant vs. control for $p < 0.05$).

substance	concentration [µM]	% of control [mean ± SD]
control	-	100
Aldosterone	100	110 ± 11
Aldosterone	10	129 ± 15
Androsterone	100	145 ± 20*
Androsterone	10	105 ± 9
Cortisone	100	107 ± 13
Cortisone	10	125 ± 15
Estrone	100	68 ± 8*
Estrone	10	87 ± 7
Estriol	100	43 ± 10*
Estriol	10	96 ± 5
β-estradiol	10	35 ± 4*
Hydrocortisol	100	83 ± 15
Hydrocortisol	10	92 ± 7
Hydroxyprogesterone	10	168 ± 12*
Mifepristone	100	7 ± 1*
Mifepristone	10	26 ± 4*
Pregnenolone	50	129 ± 4
Pregnenolone	10	171 ± 27*
Progesterone	100	66 ± 5
Progesterone	10	186 ± 14*
Testosterone	100	27 ± 4*
Testosterone	10	84 ± 8
Conjugates		
Estrone-3-sulfate	10	43 ± 5*
Estradiol-glucuronide	10	106 ± 3
DHEAS	10	70 ± 2*

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Pregnenolone sulfate	10	28 ± 7*
Pregnenolone acetate	10	83 ± 15

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Table 2. Effect of different steroids on the OATP2B1 mediated DHEAS uptake. OATP2B1-transfected MDCKII cells were incubated with [^{3}H] DHEAS (1µCi/ml) for 10 min in the presence of indicated compounds (n = 3; *significant vs. control for $p < 0.05$).

substance	concentration [µM]	% of control [mean ± SD]
control	-	100 ± 3
Androsterone	100	121 ± 5
Androsterone	10	159 ± 27*
Estrone	100	283 ± 43*
Estrone	10	288 ± 57*
Estriol	100	78 ± 13
Estriol	10	108 ± 8
β-estradiol	10	158 ± 15*
Hydrocortisol	100	158 ± 35
Hydrocortisol	10	93 ± 11
Hydroxyprogesterone	100	241 ± 6*
Hydroxyprogesterone	10	209 ± 13*
Mifepristone	100	55 ± 15*
Mifepristone	10	69 ± 10*
Pregenolone	50	179 ± 14*
Pregenolone	10	184 ± 12*
Progesterone	100	303 ± 36*
Progesterone	10	346 ± 3*
Testosterone	100	55 ± 4*
Testosterone	10	75 ± 6*
Conjugates		
Estrone-3-sulfate	100	64 ± 7*
Estrone-3-sulfate	10	92 ± 10
Estradiol-glucuronide	10	95 ± 13
Pregnenolone sulfate	100	43 ± 7*

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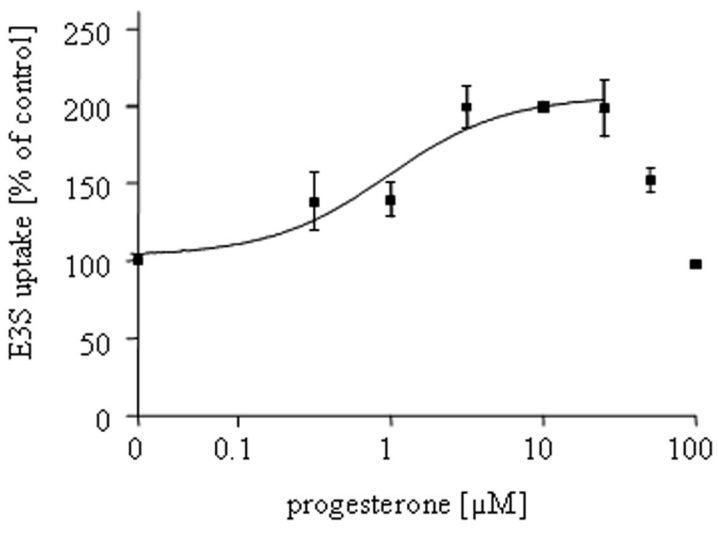
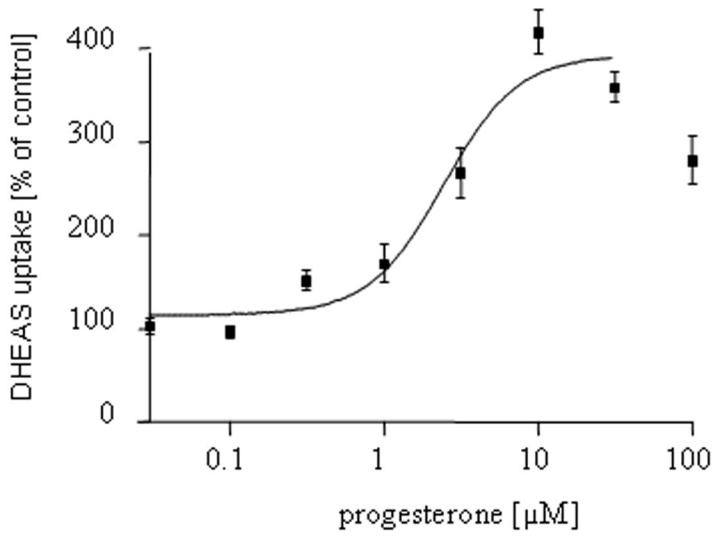
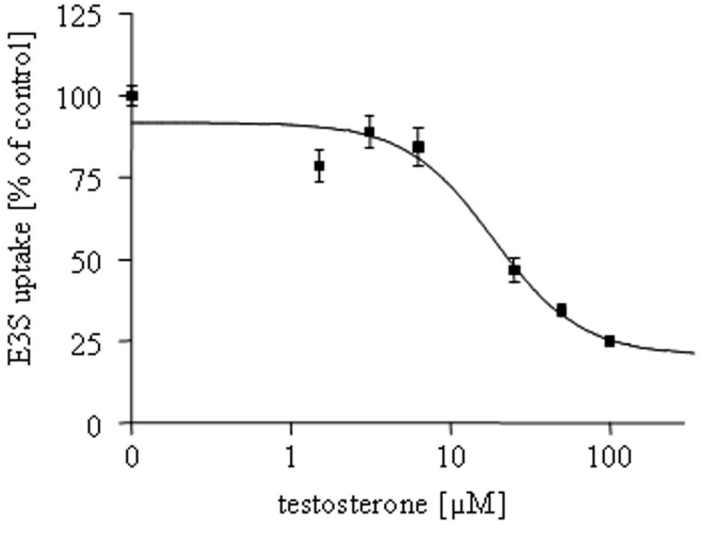
Table 3. Uptake of [^{3}H] E3S (20 nM, 1 µCi/ml), [^{3}H] DHEAS (17 nM, 1 µCi/ml), [^{3}H] progesterone (20 nM, 1 µCi/ml) and [^{3}H] testosterone (20 nM, 1 µCi/ml) into OATP2B1-transfected and non-transfected MDCKII cells. Cells were incubated for 15 min with the respective substance (n = 3; *significant vs. MDCKII for $p < 0.05$).

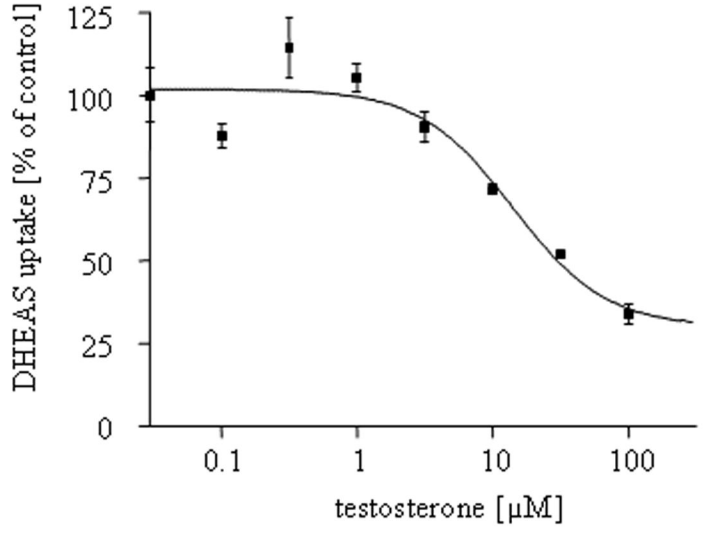
	MDCKII mean ± SD [fmol/min/mg]	OATP2B1 mean ± SD [fmol/min/mg]	OATP2B1 specific
E3S	11.1 ± 1.1	301 ± 33*	290
DHEAS	11.4 ± 1.9	36.3 ± 3.5*	24.9
progesterone	488 ± 20	354 ± 44*	-135
testosterone	67.3 ± 5.4	56.1 ± 9.9	-11.2

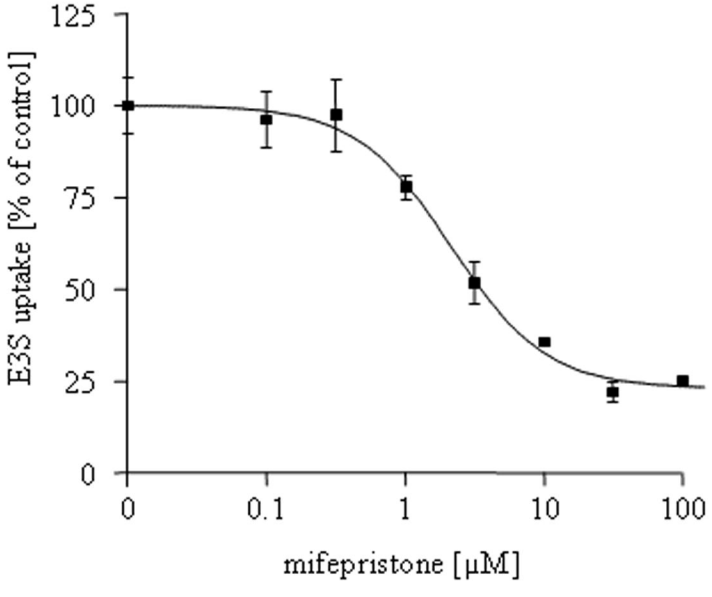
Figure 1











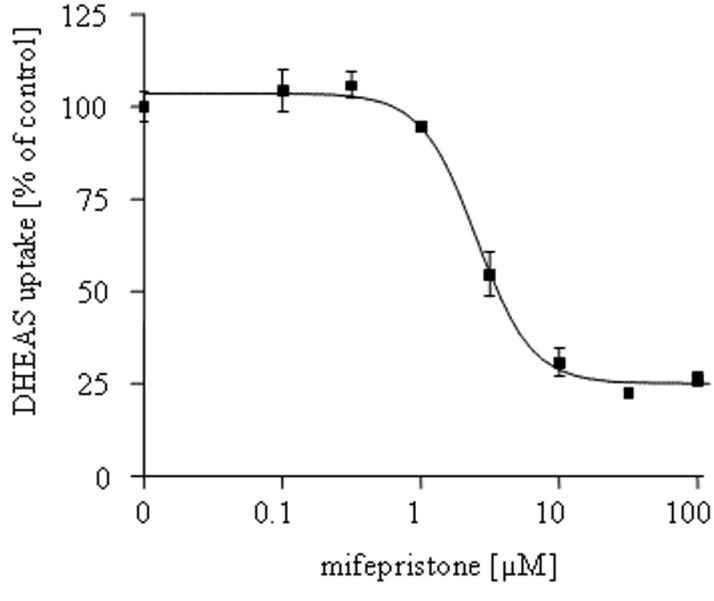


Figure 2

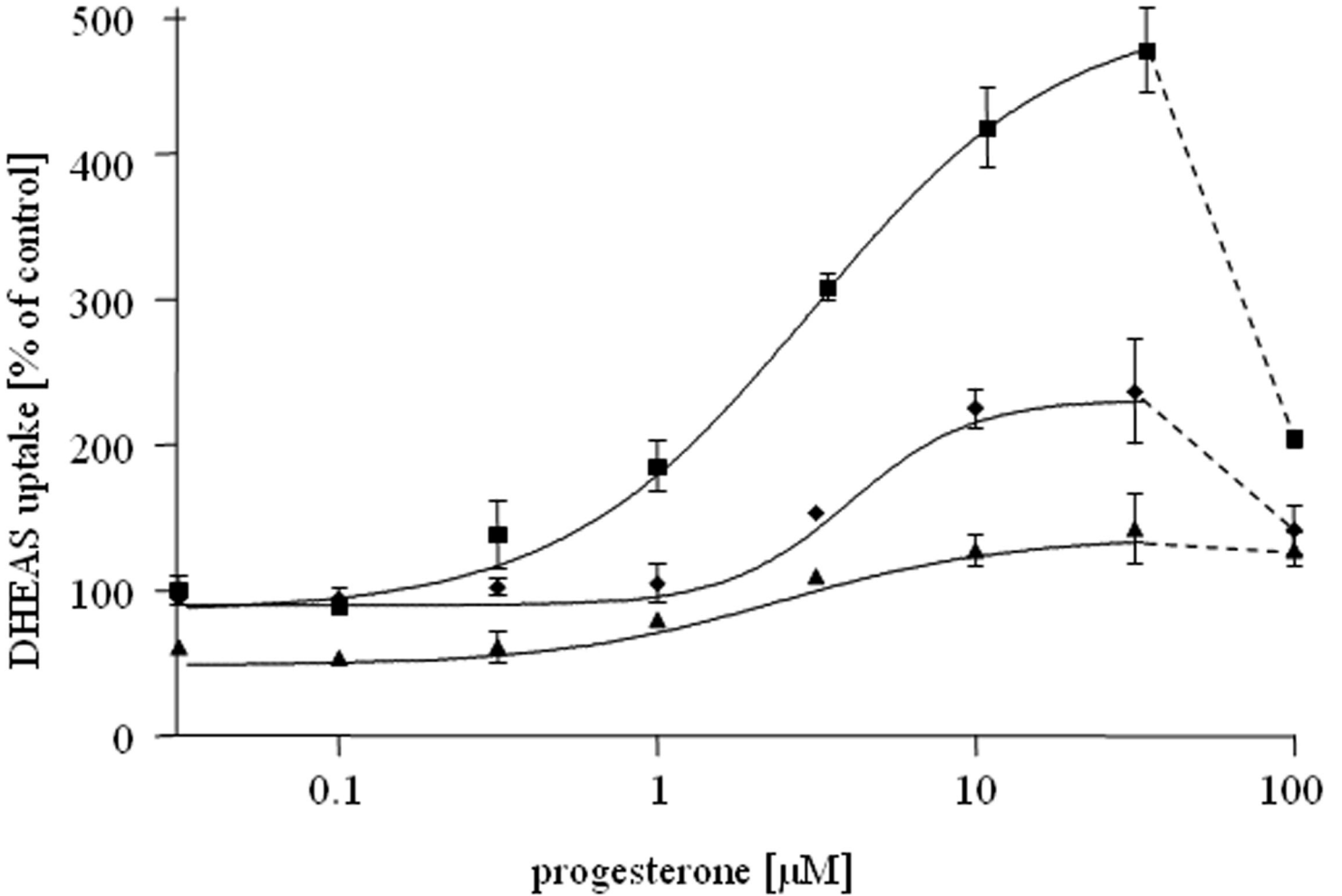

500
400
300
200
100
0
DHEAS uptake [% of control]
0.1
1
10
100
progesterone [µM]

Figure 3

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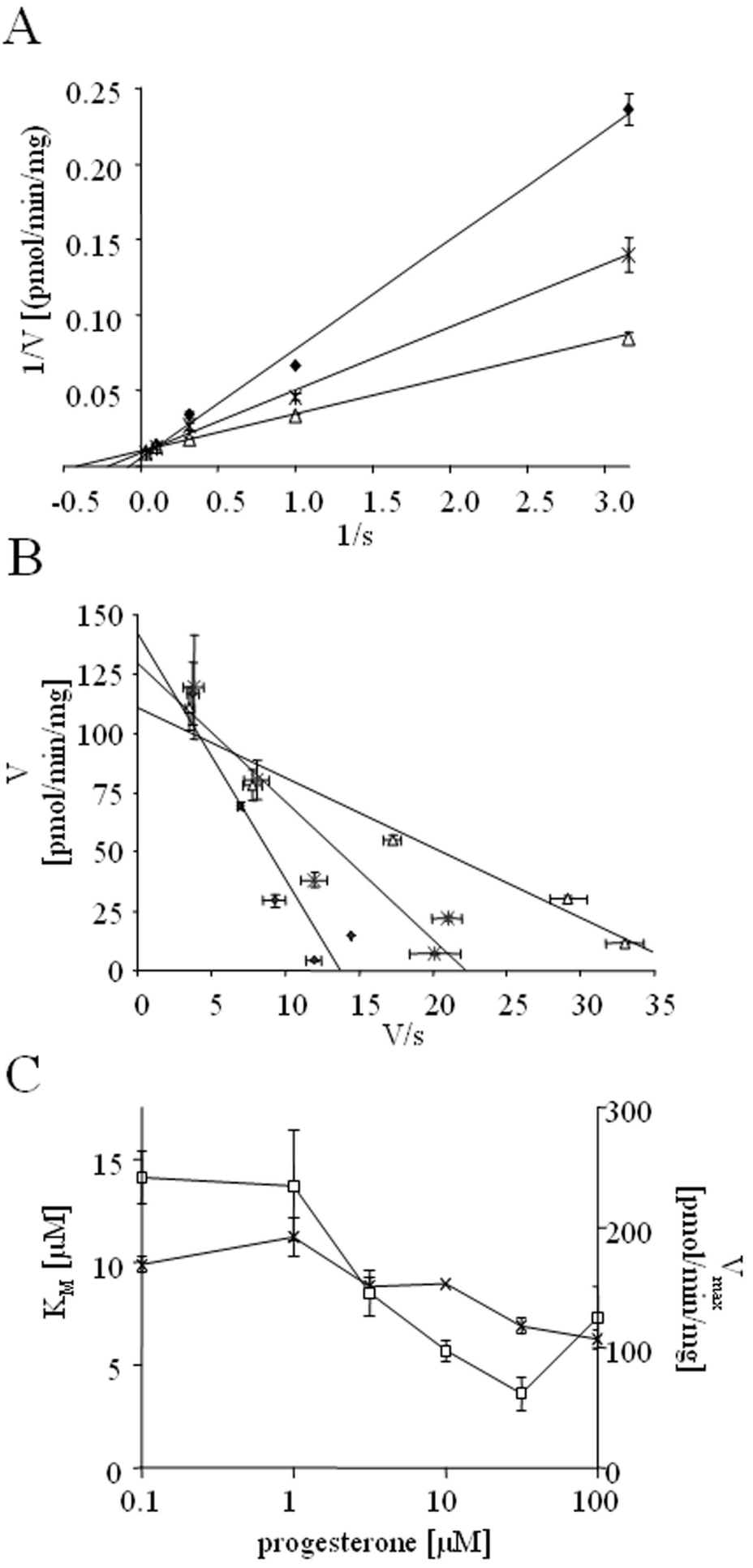

Figure 4

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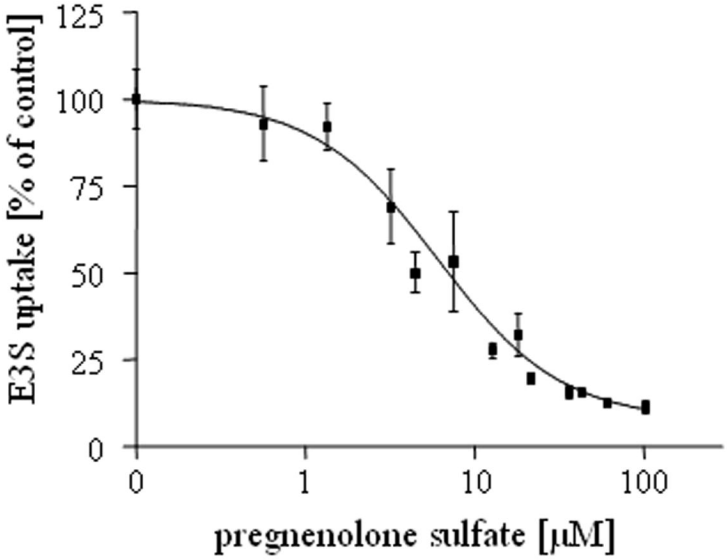

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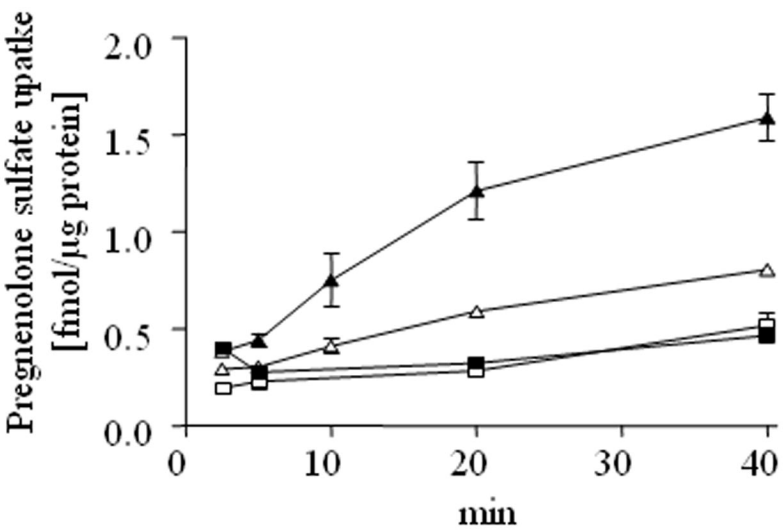

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