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Functional characterization of active transport of progesterone to adrenal cells

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Abstract

The characterization of the transport mechanism of progesterone, which is one of the neutral steroids in the adrenal cells, has been studied by the examination of progesterone uptake into the monolayers of SW-13 cells (a human adrenal adenocarcinoma cell line). The uptake of [^{3}H]progesterone at a tracer concentration (1 nM) exhibited temperature, pH and sodium dependency. According to kinetic analysis of the concentration dependence, the uptake of progesterone involves saturable and non-saturable processes. The uptake for the saturable process, which gave K_t values (half-saturation concentration) of $4.7 \pm 8.7\ \mu$M, was inhibited by metabolic inhibitors and amino-acid modifiers but not by endocytosis inhibitors or substrates for known transporters. The uptake of progesterone was also inhibited by several neutral steroids but not by anionic steroids. The inhibition by both β-estradiol and estriol was competitive. The uptake of progesterone by the adrenal cells might be at least partially accounted for by a specific carrier-mediated transport mechanism generated by sodium ions and an electrochemical mechanism.

Introduction

Steroid hormones are biosynthesized and perform biological functions in the endocrine organs such as the ovary, testis and adrenal gland in mammals. It is known that several functions that could strictly discriminate the structural characters of steroid hormones, such as receptors (Brann et al 1995; Raza et al 2001), metabolic enzymes (Casey et al 1983; Eacho & Colby 1985) and binding sites (Trueba et al 1990; Andrés et al 1997; Rae et al 1998), exist in the cells constitutively and on the membrane surface of the tissues. On the other hand, several reports have indicated that not only steroid hormones such as estrone, estradiol, estriol (Ullberg & Bengtsson 1963; Tsuchiya et al 1997) and progesterone (Maeyama et al 1969), but also xenobiotic drugs which have steroid structures (Miyamoto et al 1991), are distributed to the adrenal and ovary rather than to the other tissues when these steroids are administered to experimental animals and man. It has been mentioned that most steroid hormones are transported to the cells by passive diffusion because of their high hydrophobicity. Moreover, the steroids might accumulate in the endocrine organs by metabolic enzymes and binding sites that are described above. However, it is thought to be more appropriate that these endocrine organs have other active mechanisms, such as mediation of transporter(s) and pump(s), to concentrate steroids in the organs so as to aid the function of the receptors and metabolic enzymes.

This study was intended to characterize the transport mechanism of neutral steroids to the adrenal cells by examining the uptake of progesterone to the human adrenal adenocarcinoma cell line, SW-13. We chose progesterone as the substrate for this study, since it was first reported that progesterone was distributed in the adrenal gland when it was injected into the human fetus (Maeyama et al 1969); secondly, it has moderate solubility for the uptake study; thirdly, radio-labelled progesterone with high radioactivity is commercially available; and finally, progesterone analogues (progesterone-agonistic reagents) were recently noted as drugs for hormone replacement therapy (Sitruk-Ware 2002).

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Materials and Methods

Materials

SW-13 cells were obtained from the American Type Culture Collection (Rockville, MD). [^{3}H]Progesterone (1776 GBq mmol^{-1}) and [^{14}C]antipyrine (1924 MBq mmol^{-1}) were purchased from NEN Life Science Products Inc. (Boston, MA) and American Radiolabeled Chemicals Inc. (St Louis, MO), respectively. The radiochemical purity of these compounds was above 99% as determined by high-performance liquid chromatography and was maintained during the experiment period at −20 °C. Estrone, β-estradiol, estriol, *cis*-androsterone, 5α-dihydrotestosterone, sodium azide, (±)-dithiothreitol (DTT), *N*-ethylmaleimide (NEM) and breferdine A were purchased from Wako Pure Chemical Industries (Osaka, Japan). Progesterone, 17α-hydroxyprogesterone, dehydroepiandrosterone, dehydrotestosterone, cortisone, corticosterone, pregnenolone, estradiol-3-sulfate, estradiol-17-glucuronide, estriol-3-sulfate, estriol-17-glucuronide, cholic acid, taurocholic acid, diethylstilbestrol, diethylpyrocarbonate (DEPC), dancylcadaverine, verapamil, ciclosporin, digoxin, phenylalanine, glycylglycine (Gly-Gly), L-lactic acid, *p*-aminohippuric acid, L-carnitine, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) and Dulbecco's Modified Eagle Medium (DMEM) were purchased from Sigma Chemicals (St Louis, MO), while tetraethylammonium chloride (TEA) was purchased from Tokyo Chemical Industry (Tokyo, Japan). Testosterone, dinitrophenol (DNP) and ouabain were purchased from Nacalai Tesque (Kyoto, Japan). Hydrocortisone was purchased from Merck (Darmstadt, Germany). Fetal calf serum and the penicillin (1000 IU mL^{-1})–streptomycin (1000 μg mL^{-1}) mixed solution were obtained from Invitrogen (Carlsbad, CA). All other chemicals were of reagent grade or the highest purity commercially available.

Krebs-Ringer phosphate buffer (KRPB (mM): 128 NaCl, 4.7 KCl, 1.25 $MgCl_2$ $6H_2O$, 1.25 $CaCl_2$ $2H_2O$, 10 NaH_2PO_4 $2H_2O$; adjusted to pH 8.5, 7.4, 5.0 or 6.0 by 1 M NaOH or 1 M HCl), sodium-free Krebs-Ringer phosphate buffer (sodium-free KRPB (mM): 128 choline chloride, 4.7 KCl, 1.25 $MgCl_2$ $6H_2O$, 1.25 $CaCl_2$ $2H_2O$, 10 KH_2PO_4 $2H_2O$; adjusted to pH 7.4 by 1 M KOH) and chloride-free Krebs-Ringer phosphate buffer (chloride-free KRPB (mM): 128 Na gluconate, 4.7 KH_2PO_4, 1.25 $Mg(OH)_2$, 1.25 Ca gluconate H_2O, 10 NaH_2PO_4 $2H_2O$; adjusted to pH 7.4 by 1 M NaOH) were used after filtration through a microfilter (MILLEX-LG 0.2 μm, MILLIPORE, or filter unit 0.2 μm NALGENE).

Cell culture and uptake experiments

The SW-13 cells used in this study were between passages 37 and 93, and were cultured (2.5×10^5 cells/well) on Cupcluster™ with 12 wells, 11.2 mm in diameter (Costar, Bedford, MA), in DMEM containing 10% fetal calf serum, 10 IU mL^{-1} penicillin and 10 μg mL^{-1} streptomycin. All cells were cultivated for 4 days in wells and used for the uptake experiments.

Details for the condition of each experiment are described in the legends to the figure and table footnotes. A typical experiment was as follows. The cells grown on the well were washed twice with warmed (37 °C) KRPB. To initiate uptake, a test solution (pH 8.5, 7.4, 6.0 or 5.0; 37 °C) containing [^{3}H]progesterone (1 nM) was loaded in the well of a cell insert. At designated times, solution was removed from the well and the cells were washed twice with ice-cold saline. For the quantification of the substrate in the cells, these cells were solubilized with 0.5 mL of Solvable™ (NEN, Boston, MA). The radioactivity was determined by a liquid scintillation counter (LS6000TA; Beckman, Fullerton, CA). Cellular protein was measured using DC protein assay kit™ (BIO-RAD) with bovine serum albumin as the standard. The amount of the radiolabelled compound taken up by the cells was expressed as cell/medium ratio (C/M ratio; μL (mg protein)$^{-1}$), which was obtained by dividing the amount taken up (μg) by the initial progesterone concentration in the test solution (μg μL^{-1}) and the amount of cellular protein (mg protein). In the concentration-dependency study, the appropriate amount of non-labelled progesterone was added in the test solution containing a tracer amount of [^{3}H]progesterone (1 nM). In the inhibition studies, several experiments were performed in medium containing a maximum of 1% dimethyl sulfoxide (DMSO) to dissolve the inhibitors. This concentration of DMSO did not interfere with the uptake of substance. [^{14}C]Antipyrine was used as a standard substrate for passive diffusion in the uptake studies, which were performed for 30 s at 37 °C. Each result, except for the uptake coefficient, represents the mean ± s.d of three experiments using the same cultivation of SW-13 cells.

Data analysis

The uptake coefficient (μL s^{-1} (mg protein)$^{-1}$) was evaluated from the slope of the initial linear portion of the C/M ratio (μL (mg protein)$^{-1}$)-vs-time (s) curves by a linear regression analysis. From the concentration dependency study, the uptake (pmol/mg protein/20 s) was obtained by multiplying the C/M ratio at 20 s after the uptake started (μL/mg protein/20 s) and the initial progesterone concentration in the test solution. To estimate kinetic parameters for the saturable uptake by SW-13 cells, the uptake rate (J; pmol/mg protein/20 s) was fitted to Equation 1, consisting of both saturable and the non-saturable linear terms, by using a non-linear least-squares regression analysis program, WinNonlin™ (SCI, Apex, NC).

$$J = J_{max} \times S/(K_t + S) + k_d \times S \quad (1)$$

where J_{max} is the maximum uptake rate (pmol/mg protein/20 s) for the carrier-mediated process, S is the concentration of the substrate (μM), K_t is the half-saturation concentration (μM), and k_d is the first-order rate constant (μL/mg protein/20 s). A statistical analysis was performed by using Student's two-tailed *t*-test. A difference between the means was considered to be significant when P was less than 0.05.

Results

Time and temperature dependence of progesterone uptake

Figure 1 shows the time course and the effect of temperature on the uptake of progesterone into SW-13 cells. The uptake of [^{3}H]progesterone at 37 °C rapidly and linearly increased with time for 20 s, then slowly increased. The uptake coefficient of [^{3}H]progesterone at 37 °C for the initial uptake (Figure 1, inset), 0.459 μL s^{-1}(mg protein)$^{-1}$, was higher than that observed at 4 °C (0.212 μL s^{-1} (mg protein)$^{-1}$). Accordingly, in the following experiments, the uptake studies for progesterone were performed for 20 s at 37 °C.

pH and ion dependence

The uptake of [^{3}H]progesterone by SW-13 cells declined following decrease of pH in the acidic range (Figure 2); the uptake for 20 s at pH 6.0 and 5.0 was 79.5 ± 4.2% and 71.1 ± 2.0% of the control (at pH 7.4), respectively (both $P < 0.05$). The uptake at pH 8.0 was 93.7 ± 6.8% of the control ($P > 0.05$). The uptake declined under the sodium-free condition (71.8 ± 6.7% of the control, $P < 0.05$), whereas the chloride-free condition had no effect on the progesterone uptake (97.8 ± 8.1% of the control value, $P > 0.05$).

Concentration dependence

Figure 3 shows the relationship between the initial uptake rate of [^{3}H]progesterone and its concentration in the medium from 1 nM to 10 μM. These results indicate that the uptake consists of a saturable process and a non-saturable process. The C/M ratio at 20 s after starting the uptake was 15.9–16.7 μL/mg protein/20 s over the concentration range 1.0 nM to 1.0 μM, and steeply decreased to 13.6–14.3 μL/mg protein/20 s at concentrations higher than 3.0 μM. A kinetic analysis of the concentration-dependent uptake of progesterone gave J_{max}, K_t and k_d

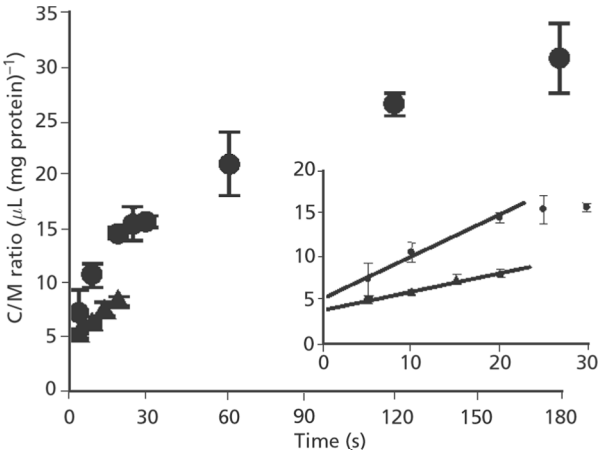

Figure 1 Time courses and temperature dependence of [^{3}H]progesterone uptake by SW-13 cells. The uptake of [^{3}H]progesterone (1 nM) was measured from 5 to 180 s during incubation at 37 °C (●) or 4 °C (▲) by incubating SW-13 cells in KRPB (pH 7.4). Each uptake coefficient was obtained by dividing the slope of the initial linear portion of the C/M ratio–time curve (inset). Each point represents mean ± s.d. of three experiments.

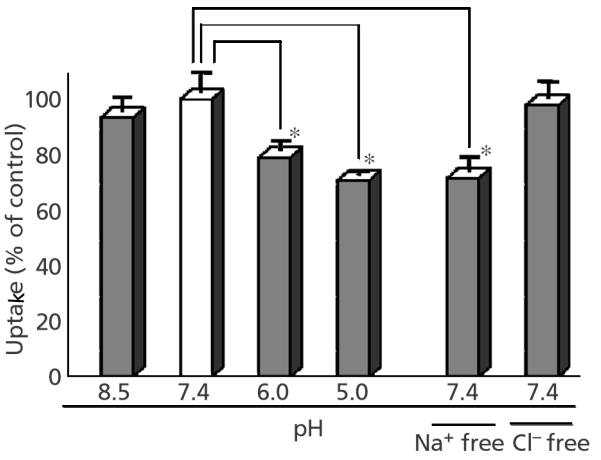

Figure 2 Effect of medium on [^{3}H]progesterone uptake by SW-13 cells. The uptake of [^{3}H]progesterone (1 nM) was measured at 20 s during incubation at 37 °C by incubating SW-13 cells in KRPB (pH 8.5, 7.4 (control), 6.0 or 5.0), sodium-free KRPB (pH 7.4) or chloride-free KRPB (pH 7.4). Each value represents the mean ± s.d. of three experiments and is expressed as a percentage of the control. *$P < 0.05$ vs control (Student's t-test).

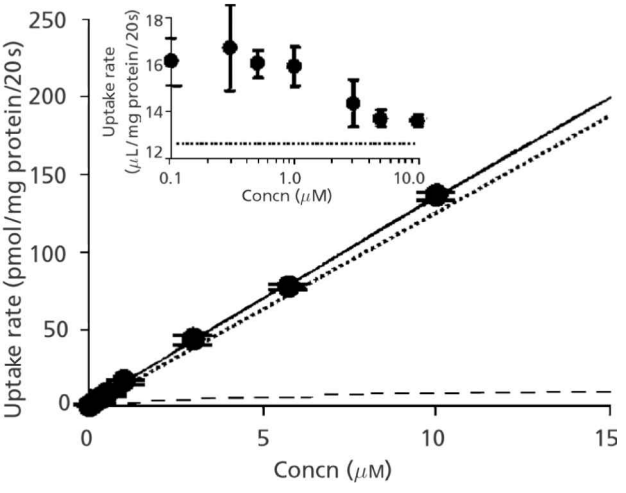

Figure 3 Concentration dependence of [^{3}H]progesterone uptake by SW-13 cells. The uptake of [^{3}H]progesterone (1 nM) was measured at 20 s during incubation at 37 °C by incubating SW-13 cells in KRPB (pH 7.4). Inset figure represents the relationship between C/M ratio at 20 s after starting uptake and the progesterone concentration. The dashed line and dotted line represent the uptake rate for the saturable and non-saturable component, respectively, calculated from the kinetic parameters obtained as mentioned in the Results. Each point represents the mean ± s.d. of three experiments.

values of 12.3 ± 22.0 pmol/mg protein/20 s, 4.7 ± 8.7 μM and 12.7 ± 1.0 μL/mg protein/20 s, respectively.

Inhibitory effect of various compounds on progesterone uptake

The uptake of [^{3}H]progesterone by SW-13 cells was significantly reduced by ATPase inhibitors, such as sodium azide, DNP and ouabain (71.6–81.2% of control), and by amino-acid modifiers, such as DEPC, DTT and NEM (71.6–84.8% of control) (Table 1). Neither the metabolic inhibitors nor amino-acid modifiers had any effect on the uptake for 30 s of [^{14}C]antipyrine, which reflects the passive diffusion. The uptake of [^{3}H]progesterone did not change in the presence of the substrates or inhibitors of p-glycoprotein (verapamil, ciclosporin and digoxin) (Tamai & Tsuji 2000) and the inhibitors of endocytosis (breferdine A and dancylcadaverine). Moreover, the uptake of [^{3}H]progesterone did not change in the presence of the typical substrates for known transporters – phenylalanine for the amino-acid transporter (Hidalgo & Borchardt 1990), Gly-Gly for the peptide transporter (Matsumoto et al 1994), TEA (Okuda et al 1996), L-carnitine (Nezu et al 1999) for the organic cation transporter, L-lactic acid (Ogihara et al 1996) and *p*-aminohippuric acid (Kanai et al 1996) for the monocarboxylate transporter and/or organic anion transporter and DIDS, a specific inhibitor for the organic anion transporter.

Table 1 Inhibitory effect of various compounds on [^{3}H]progesterone and [^{14}C]antipyrine uptake by SW-13 cells.

Compound	Concn (μM)	Uptake (% of control)[a]	
		[^{3}H]progesterone	[^{14}C]antipyrine
Sodium azide	10000	81.2 ± 0.6*	99.3 ± 3.5
DNP	1000	71.6 ± 7.4*	114.4 ± 12.4
Ouabain	1000	79.1 ± 4.8*	111.6 ± 10.1
DEPC	200	84.8 ± 2.2*	109.6 ± 12.2
DTT	10000	71.6 ± 8.4*	99.6 ± 2.1
NEM	500	79.7 ± 4.1*	99.1 ± 4.2
Verapamil	10	99.3 ± 8.4	
Ciclosporin	100	100.8 ± 4.6	
Digoxin	150	105.5 ± 13.1	
Breferdine A	20	108.2 ± 5.5	
Dancylcadaverine	50	97.3 ± 2.8	
Phenylalanine	10000	101.7 ± 4.9	
Gly-Gly	10000	98.3 ± 3.7	
TEA	500	88.1 ± 6.9	
L-Lactic acid	10000	107.3 ± 7.7	
p-Aminohippuric acid	10000	100.9 ± 14.6	
L-Carnitine	500	106.1 ± 2.4	
DIDS	1000	91.4 ± 6.9	

Uptake of [^{3}H]progesterone (1 nM) and [^{14}C]antipyrine (1 μM) was measured at 20 s and 30 s, respectively, at 37 °C by incubating SW-13 cells in KRPB (pH 7.4) containing each compound. Several experiments were performed in medium containing a maximum of 1% dimethyl sulfoxide. [a]Each data point represents the mean ± s.d. of three experiments and is expressed as a percentage of the control. *$P < 0.05$ vs control (Student's *t*-test).

Inhibitory effect of steroids on progesterone uptake

The uptake of [^{3}H]progesterone by SW-13 cells was significantly reduced in the presence of neutral steroids such as estrone, β-estradiol, estriol, testosterone, 17α-hydroxyprogesterone, *cis*-androsterone, dehydroepiandrosterone, 5α-dihydrotestosterone, dehydrotestosterone, cortisone, corticosterone and pregnenolone, being 66.4–86.0% of control ($P < 0.05$, Table 2). The inhibitory effect of hydrocortisone was not significant but was notable (79.0% of control). The uptake of [^{3}H]progesterone did not change in the presence of cholic acid, taurocholic acid and the anionic conjugates (sulfate and glucronide) of estriol and estradiol, including a substrate of multi-drug resistance protein 1 or 2 (MRP1 or 2), estradiol-17-glucuronide (König et al 1999). Diethylstilbestrol, a synthetic hormone which does not have steroid structure, had no effect on progesterone uptake.

Kinetic analysis of inhibitory effect

To study the mechanism of inhibition of progesterone transport by steroids, the inhibitory effects of oestrogens on [^{3}H]progesterone were kinetically analysed. Figure 4 shows Lineweaver–Burk plots for the effect of β-estradiol and estriol on the progesterone uptake rate after subtraction of the non-saturable component. Competitive inhibition was observed and the evaluated inhibition constant, K_i, of β-estradiol and estriol was 8.7 and 3.6 μM, respectively.

Discussion

In this study, characterization of the transport mechanism of neutral steroids to the adrenal cells was performed by examining the uptake of progesterone across the monolayers of SW-13 cells. The uptake of [^{3}H]progesterone at neutral pH was faster than that observed under acidic conditions, and was reduced in the absence of sodium ion. Moreover, the uptake of [^{3}H]progesterone exhibited a temperature dependency and was inhibited by ATPase inhibitors. Accordingly, the uptake of [^{3}H]progesterone by SW-13 cells is pH- and sodium-ion-dependent, and ATP might be one of the driving forces in this process. Furthermore, the progesterone uptake mechanism existed on the surface of the cell membrane, such as transporter(s), but was not endocytosis since the uptake process was inhibited by amino-acid modifiers and not by endocytosis inhibitors.

In terms of affinity, the apparent uptake of progesterone characterized in this study was strikingly different from the receptors and binding sites reported previously. According to the kinetic analysis of the concentration-dependent uptake of progesterone, the K_t value of 4.7 μM

Table 2 Inhibitory effect of steroid hormones on of [^{3}H]progesterone uptake by SW-13 cells.

Steroid hormone	Uptake (% of control)[a]
Estrone	71.0 ± 8.7*
β-Estradiol	80.6 ± 3.5*
Estriol	76.6 ± 7.8*
Testosterone	77.1 ± 2.2*
17α-Hydroxyprogesterone	69.4 ± 6.4*
cis-Androsterone	86.0 ± 1..9*
Dehydroepiandrosterone	82.6 ± 4.4*
5α-Dihydrotestosterone	66.4 ± 6.3*
Dehydrotestosterone	80.3 ± 3.5*
Hydrocortisone	79.0 ± 12.8
Cortisone	83.2 ± 1.6*
Corticosterone	69.0 ± 1.6*
Pregnenolone	83.3 ± 6.3*
Estradiol-3-sulfate	105.8 ± 4.6
Estradiol-17-glucuronide	107.8 ± 8.7
Estriol-3-sulfate	101.3 ± 11.8
Estriol-17-glucuronide	108.1 ± 3.4
Cholic acid	107.5 ± 15.0
Taurocholic acid	96.6 ± 5.3
Diethylstilbestrol	91.4 ± 11.3

Uptake of [^{3}H]progesterone (1 nM) was measured at 20 s at 37 °C by incubating SW-13 cells in KRPB (pH 7.4) containing each steroid hormone (100 μM). All experiments were performed in medium containing a maximum of 1% dimethyl sulfoxide. [a]Each data point represents the mean ± s.d. of three experiments and is expressed as a percentage of the control. *$P < 0.05$ vs control (Student's *t*-test).

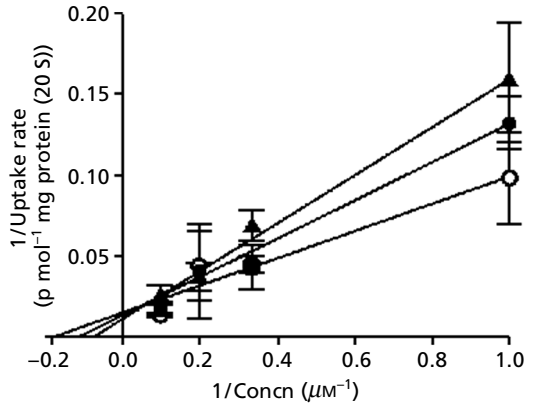

Figure 4 Lineweaver–Burk plots for progesterone uptake by SW-13 cells. The uptake was measured in the absence (○) or presence of 5 μM β-estradiol (●) and estriol (▲), respectively. The incubation conditions were identical to those described in the legend to Figure 3. Each point represents the mean ± s.d. of three experiments.

(10^{-6} M) was dramatically higher than the K_d values for the steroid receptors (10^{-10}–10^{-11} M) (Brann et al 1995; Raza et al 2001) including σ-receptors (10^{-8}M) (Ganapathy et al 1999) and K_d values for the steroid hormone binding sites (10^{-8}–10^{-9} M) (Trueba et al 1990; Andrés et al 1997; Rae et al 1998), which existed in the cells and on the membrane surface of the tissues. Alléra & Wildt (1992) performed active corticosterone transport mediated by a putative, plasma membrane-inserted carrier for glucocorticoids. They found that the corticosterone uptake was a saturable process with three significantly different K_d values of 1.3, 4.7 and 17.3 nM, which were strikingly lower than the K_t value of the progesterone uptake to SW-13 cells.

The uptake of [^{3}H]progesterone was inhibited by several neutral steroids, not by the anionic steroid derivatives. The competitive inhibition between progesterone and oestrogens (β-estradiol and estriol) indicates that progesterone and these oestrogens share a common transport system in adrenal cells. Accordingly, it was thought that the substrate-recognizable spectrum of the progesterone uptake mechanism is specific for neutral steroids, but non-specific among them, whereas the reported steroid receptors, metabolic enzymes and binding sites critically discriminate between the steroids as substrates (Casey et al 1983; Eacho & Colby 1985; Trueba et al 1990; Brann et al 1995; Andrés et al 1997; Rae et al 1998; Raza et al 2001). Sakai et al (1992) showed that an anionic steroidal drug, sodium prasterone sulfate, was not distributed to the adrenal when administered to rats, which supports our results. Although diethylstilbestrol, an artificial non-steroidal hormone, was distributed to the adrenal when administered to experimental animals (Bengtsson & Ullberg 1963), progesterone uptake was not inhibited by this drug in this study. This drug might be distributed to the adrenal by another mechanism. On the other hand, since cortisone that was not distributed to the adrenal (Hanngren et al 1964) inhibited the progesterone uptake, this steroid might take the effect of an inhibitor but not of a substrate, for the progesterone uptake system.

The uptake of [^{3}H]progesterone did not change in the presence of typical substrates or inhibitors for known transporters, such as amino-acid transporter (Hidalgo & Borchardt 1990), peptide transporter (Matsumoto et al 1994), organic cation transporter (Okuda et al 1996), monocarboxylate transporter (Ogihara et al 1996), organic anion transporter (Kanai et al 1996) and p-glycoprotein (Tamai & Tsuji 2000). In particular, the uptake was not affected by the substrate of organic cation/carnitine transporter 2 (OCTN2), L-carnitine (Nezu et al 1999), and that of MRP1 or MRP2, estradiol-17-glucuronide (König et al 1999). Therefore, it is thought that the uptake of progesterone is not concerned with these transporters.

Conclusions

In this study, we have clarified that the uptake of progesterone by adrenal cells is performed by the sodium and pH-dependent and carrier-mediated uptake system specific for neutral steroids. The uptake of neutral steroids by the adrenal cells might be at least partially accounted for by the specific carrier-mediated transport mechanism enhanced by sodium ion at neutral pH. Furthermore, it is suggested that several neutral steroids share a common transport system with neutral steroids. Besides, The K_t value of 4.7 μM was remarkably higher than the human blood concentration of progesterone (10^{-7}–10^{-8} M)

(Johansson 1969). This causes the ambiguous discussion about the physiological function of the transport system. It is thought rational that the endocrine organs have an efficient mechanism(s) on the membrane surface to accumulate steroids in the organs from the blood circulation system rather than by passive diffusion. The receptors and metabolic enzymes for steroid hormones in the organs and the active transport system may cooperate to perform steroid biological functions and metabolism. It is also reported that many steroidal xenobiotic drugs are distributed to the adrenal gland when these drugs are administered to mammals. Accordingly, it is suggested that these drugs are transported to the adrenal by a common transport system for neutral steroids. Further studies are needed to clarify the physiological roles of the uptake mechanism of steroids to these organs.

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