Transport of carnitine and acetylcarnitine by carnitine/organic cation transporter (OCTN) 2 and OCTN3 into epididymal spermatozoa

Daisuke Kobayashi, Ikumi Tamai1, Yoshimichi Sai, Kazuhiro Yoshida, Tomohiko Wakayama2, Yasuto Kido, Jun-ichi Nezu3, Shoichi Iseki2 and Akira Tsuji

Division of Pharmaceutical Sciences, Department of Molecular Biopharmaceutics, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan, 1Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamasaki, Noda, Chiba 278-8510, Japan, 2Department of Histology and Embryology, Graduate School of Medical Science, Kanazawa University, Takara-machi, Kanazawa 920-8640, Japan and 3Chugai Pharmaceutical Co. Ltd, Shizuoka, Japan

Correspondence should be addressed to A Tsuji; Email: tsuji@kenroku.kanazawa-u.ac.jp

Abstract

Carnitine and acetylcarnitine are important for the acquisition of motility and maturation of spermatozoa in the epididymis. In this study, we examined the involvement of carnitine/organic cation transporter (OCTN) in carnitine and acetylcarnitine transport in epididymal spermatozoa of mice. Uptake of both compounds by epididymal spermatozoa was time-dependent and partially Na+-dependent. Kinetic analyses revealed the presence of a high-affinity transport system in the spermatozoa, with $K_m$ values of 23.6 and 6.57 mM for carnitine and acetylcarnitine respectively in the presence of Na+. Expression of OCTN2 and OCTN3 in epididymal spermatozoa was confirmed by immunofluorescence analysis. The involvement of these two transporters in carnitine and acetylcarnitine transport was supported by a selective inhibition study. We conclude that both Na+-dependent and -independent carnitine transporters, OCTN2 and OCTN3, mediate the supply of carnitine and acetylcarnitine to epididymal spermatozoa in mice.


Introduction

Acquisition of motility and maturation of spermatozoa occur during passage through the epididymal tract (Dacheux & Paquingnon 1980, Jeulin & Lewin 1996). Carnitine and acetylcarnitine are essential nutrients for sperm maturation (Casillas & Chaipayungpan 1979, Hinton et al. 1981) and are present in epididymal plasma at concentrations of 1–63 mM (Marquis & Fritz 1965, Casillas 1972, Hinton et al. 1979, Jeulin & Lewin 1996), which is significantly higher than that in blood plasma (about 50 μM). Such high concentrations of carnitine and acetylcarnitine in epididymal plasma may be due to active supply from blood to the tissue, and indeed, the presence of transporters in the tissue has been reported (Yeung et al. 1980, Cooper et al. 1986, Radigue et al. 1996, Kobayashi et al. 2005). The concentration of carnitine in epididymal plasma increases from the proximal part (caput) to the distal part (cauda) of the epididymis (Hinton et al. 1979, Casillas et al. 1984, Jeulin et al. 1987, Jeulin & Lewin 1996). Similarly, the carnitine concentration in epididymal spermatozoa increases during transition through the epididymal tract (Casillas 1973, Casillas & Chaipayungpan 1979, Casillas et al. 1984). Sperm motility is correlated with acetylcarnitine concentration in ejaculated human spermatozoa (Johansen & Bohmer 1979). These observations suggest that the concentrations of carnitine and acetylcarnitine in spermatozoa are critical for the acquisition of sperm motility. Active transport across the plasma membrane of spermatozoa would be essential, since carnitine and acetylcarnitine are zwitterionic and highly hydrophilic. Temperature-dependent transport of carnitine in ejaculated bovine spermatozoa (Deana et al. 1989) and saturable carnitine transport in bovine caput epididymal spermatozoa (Casillas 1973) and human ejaculated spermatozoa (Xuan et al. 2003) has been reported. Nevertheless, other authors have suggested that carnitine uptake occurs through passive diffusion into spermatozoa in the bovine cauda epididymides (Casillas 1973) and epididymides of the boar (Jeulin et al. 1994).

We and others have isolated carnitine/organic cation transporters (OCTNs) in humans, rats, and mice (Tamai et al. 1997, 1998, 2000, Wu et al. 1998, 1999, 2000, Nezu et al. 1999). The first member of OCTNs, OCTN1 (solute carrier (SLC)22A4), transports cationic xenobiotics, such as tetraethylammonium (Tamai et al. 1984).
1997, 2000, 2004, Yabuuchi et al. 1999) and ergothioneine (Grundemann et al. 2005), and has a low activity for carnitine transport (Yabuuchi et al. 1999, Tamai et al. 2000, Grundemann et al. 2005). OCTN2 (SLC22A5) is an Na\textsuperscript{+}-dependent, high-affinity (K_m = 4–25 μM) carnitine transporter (Tamai et al. 1998, 2000, Sekine et al. 1998, Wu et al. 1999). Human carnitine transporter CT2 (SLC22A16) and mouse OCTN3 (SLC22A21) transport carnitine with high affinity (K_m = 20 and 3 μM respectively) in a sodium-independent manner (Nezu et al. 1999, Ohashi et al. 1999, Tamai et al. 2000, Enomoto et al. 2002). On the other hand, Nakanishi et al. (2001) reported that the Na\textsuperscript{+}- and Cl\textsuperscript{−}-coupled neutral and cationic amino acid transporter ATB\textsuperscript{K,Na}\textsuperscript{+} (SLC6A14) can transport carnitine with low affinity (K_m = 0.83 mM). Furthermore, OCTN2-deficient JVS mice showed male infertility with epididymal dysfunction (Toshimori et al. 1999), and OCTN3 is selectively expressed in the male reproductive tissue of mice (Tamai et al. 2000). Xuan et al. (2003) reported the presence of proteins that react with antibodies against mouse OCTN1, OCTN2 and OCTN3 in human ejaculated spermatozoa. Based on these findings, we considered that OCTN2 and OCTN3 could contribute to carnitine and acetylcarnitine transport in epididymal spermatozoa. However, this issue remains to be clarified at the molecular level.

In this study, we examined the localization and involvement of the carnitine transporters OCTN2 and OCTN3 in the supply of carnitine and acetylcarnitine to murine spermatozoa.

Results

Time courses and ion dependence of [3H]carnitine and [3H]acetylcarnitine uptake by epididymal spermatozoa of mice

The uptakes of [3H]carnitine and [3H]acetylcarnitine by epididymal spermatozoa were measured over 30 min. Figure 1 shows that the uptake increased linearly for 5 min in both the presence and the absence of Na\textsuperscript{+}, but was slightly lower in its absence (extracellular Na\textsuperscript{+}) was replaced with N-methylglucamine; Fig. 1). Since the steady-state uptake amounts of both the compounds (Fig. 1) were higher than the intracellular water space (3.44 μl/mg protein), estimated as the difference between the uptakes of [3H]water and [14C]inulin, in epididymal spermatozoa, carnitine, and acetylcarnitine seem to be accumulated in the intracellular space of epididymal spermatozoa, suggesting the involvement of active transport. In addition, uptake clearance of acetylcarnitine was higher than that of carnitine into spermatozoa (Fig. 1). Therefore, acetylcarnitine was used as substrate in the following study of ion-dependent and inhibitory effects. Table 1 shows the sodium ion dependence of the uptake of [3H]acetylcarnitine. When Na\textsuperscript{+} was replaced with lithium or potassium, the uptake of [3H]acetylcarnitine...
Carnitine transport in spermatozoa

was increased, whereas it was decreased when Na\(^+\) was replaced with choline or N-methylglucamine. These results suggested that both Na\(^+\)-dependent and -independent transporters exist in murine spermatozoa. The effects of changing from chloride ion to other anions were also examined. Thiocyanate, nitrate, and gluconate slightly increased the uptake of \[^{3}H\]acetylcarnitine, while replacement with sulfate ion decreased the uptake.

**Concentration dependence of carnitine and acetylcarnitine uptake by murine spermatozoa**

The concentration dependence of the uptake of carnitine by murine spermatozoa is shown in Fig. 2a, and that in the case of acetylcarnitine is shown in Fig. 2b. Both uptakes were saturable. Eadie–Hofstee plots indicated the involvement of a single saturable transport system in each case (Fig. 2c and d). The kinetic parameters for carnitine uptake were calculated to be \(K_m = 23.6 \pm 14.5 \mu M\), \(V_{max} = 281 \pm 176 \text{ pmol/mg protein/3 min}\), and \(k_d = 6.55 \pm 1.33 \mu L/\text{mg protein/3 min}\), and those for acetylcarnitine were \(K_m = 6.57 \pm 1.77 \mu M\), \(V_{max} = 390 \pm 96 \text{ pmol/mg protein/3 min}\), and \(k_d = 7.99 \pm 2.63 \mu L/\text{mg protein/3 min}\) (means \(\pm\) S.E.M.). These results suggested that spermatozoa in mice have a relatively high-affinity carnitine transporter.

**Inhibitory effects of several compounds on \[^{3}H\]acetylcarnitine uptake by murine spermatozoa**

To characterize the uptake systems for acetylcarnitine in spermatozoa, we measured \[^{3}H\]acetylcarnitine uptake in the presence of several compounds. Table 2 shows that 50 \(\mu M\) γ-butyrobetaine and 500 \(\mu M\) verapamil inhibited the uptake to <50% of that in the absence of inhibitor. l-Carnitine, d-carnitine, glycinebetaine, quinidine, and tetraethylammonium (TEA) also significantly reduced \[^{3}H\]acetylcarnitine uptake by spermatozoa. Spermine, spermidine, and arginine were not inhibitory.

**Expression of OCTNs in epididymal spermatozoa of mice**

To identify the sodium-dependent and -independent carnitine transporters that showed high affinity for carnitine and acetylcarnitine at the molecular level, we employed immunofluorescence analysis, using antibodies against OCTN transporters. As shown in Fig. 3, strong signals (red) were observed in the tail of epididymal spermatozoa with anti-OCTN2 and -OCTN3 antibodies, and weak signals were observed with anti-OCTN1 antibody. No signals were detected with control IgG (Fig. 4c). A strong expression of OCTN3 was observed especially in corpus and cauda epididymal spermatozoa. The ratio of OCTN3-positive spermatozoa

![Figure 2](image-url)

**Table 2** Inhibitory effects of several endogenous compounds and xenobiotics on \[^{3}H\]acetylcarnitine uptake by spermatozoa.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration ((\mu M))</th>
<th>Relative uptake (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No inhibitors (control)</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Endogenous compounds</td>
<td></td>
<td></td>
</tr>
<tr>
<td>l-Carnitine</td>
<td>5</td>
<td>77 (\pm) 2*</td>
</tr>
<tr>
<td>d-Carnitine</td>
<td>50</td>
<td>61 (\pm) 1*</td>
</tr>
<tr>
<td>γ-Butyrobetaine</td>
<td>5</td>
<td>88 (\pm) 2*</td>
</tr>
<tr>
<td>γ-Butyrobetaine</td>
<td>50</td>
<td>44 (\pm) 1*</td>
</tr>
<tr>
<td>Glycinebetaine</td>
<td>500</td>
<td>113 (\pm) 2</td>
</tr>
<tr>
<td>Glycinebetaine</td>
<td>5000</td>
<td>74 (\pm) 2*</td>
</tr>
<tr>
<td>Spermine</td>
<td>5000</td>
<td>111 (\pm) 3</td>
</tr>
<tr>
<td>Spermidine</td>
<td>5000</td>
<td>96 (\pm) 4</td>
</tr>
<tr>
<td>Arginine</td>
<td>5000</td>
<td>135 (\pm) 2</td>
</tr>
<tr>
<td>Xenobiotics</td>
<td></td>
<td></td>
</tr>
<tr>
<td>d-Carnitine</td>
<td>5</td>
<td>89 (\pm) 2</td>
</tr>
<tr>
<td>d-Carnitine</td>
<td>50</td>
<td>79 (\pm) 1*</td>
</tr>
<tr>
<td>Quinidine</td>
<td>50</td>
<td>115 (\pm) 3</td>
</tr>
<tr>
<td>Quinidine</td>
<td>500</td>
<td>68 (\pm) 2*</td>
</tr>
<tr>
<td>Verapamil</td>
<td>50</td>
<td>69 (\pm) 4*</td>
</tr>
<tr>
<td>Verapamil</td>
<td>500</td>
<td>36 (\pm) 2*</td>
</tr>
<tr>
<td>TEA</td>
<td>5000</td>
<td>78 (\pm) 1*</td>
</tr>
</tbody>
</table>

The uptake of \[^{3}H\]acetylcarnitine (12.5 \(nM\)) by spermatozoa was measured for 3 min at 37 °C in transport buffer (pH 7.4) containing each compound. Each value represents the mean \(\pm\) S.E.M. of four determinations. *Significantly decreased from the control uptake by Student's t-test one-way ANOVA with Tukey-Kramer's post hoc test \((P<0.05)\).

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was significantly higher in corpus (92.8 ± 2.2%, n=6) and cauda (92.7 ± 1.9%, n=6) compared with caput (71.7 ± 2.4%, n=6). Figure 4 clearly shows that the localizations of OCTN2 and OCTN3 in spermatozoa were different. OCTN2 was localized to the principal piece of sperm tail, whereas OCTN3 was localized to the middle piece of the sperm tail, which lies adjacent to the nucleus (blue).

**Regional difference of acetylcarnitine uptake into epididymal spermatozoa of mice**

The transport activity of acetylcarnitine was measured in caput, corpus, and cauda spermatozoa (Fig. 5). The acetylcarnitine transport activity was extremely high in corpus spermatozoa in the absence of Na⁺ as well as in the presence of Na⁺.

**Comparison of the inhibitory effects of several compounds on [³H]carnitine uptake by OCTN2, OCTN3, and epididymal spermatozoa of mice**

To clarify the functional involvement of OCTN2 and OCTN3 in carnitine transport in epididymal spermatozoa, detailed inhibition studies were conducted. First, we required specific inhibitors for OCTN2 or OCTN3 to differentiate OCTN2- and OCTN3-mediated transports. The IC₅₀ values of six compounds (γ-butyrobetaine, butyryl-l-carnitine, pyrilamine, quinidine, TEA, and verapamil) were obtained and compared. As shown in Table 3, the IC₅₀ values of pyrilamine for OCTN2- or OCTN3-mediated carnitine uptake were 41.7 and 318 μM respectively, suggesting that pyrilamine has a higher affinity for OCTN2. In contrast, γ-butyrobetaine and butyryl-l-carnitine showed a higher affinity for OCTN3 than for OCTN2. The IC₅₀ values for OCTN2 and OCTN3 were comparable in the cases of quinidine, TEA, and verapamil. Based on these results, we chose pyrilamine and γ-butyrobetaine as specific (or more strictly, preferential) inhibitors of OCTN2 and OCTN3 respectively. The IC₅₀ values of pyrilamine and γ-butyrobetaine for carnitine uptake in epididymal spermatozoa were 208 and 30.7 μM respectively. These results suggest that both OCTN2 and OCTN3 contribute to carnitine uptake in epididymal spermatozoa.

Figure 3 Expression of OCTN proteins in murine epididymal spermatozoa. Spermatozoa isolated from caput, corpus, and cauda epididymides were incubated with affinity-purified antiserum against mouse OCTN1, OCTN2, or OCTN3 and examined by immunofluorescence microscopy. The OCTN proteins appear in red and nuclei in blue.

Figure 4 Expressions of OCTN2 and OCTN3 proteins in epididymal spermatozoa in mice. Spermatozoa isolated from cauda epididymis were incubated with affinity-purified antiserum against mouse (a) OCTN2, (b) OCTN3, or (c) normal IgG. The OCTNs appear in red and nuclei in blue.
Inhibitors

OCTN2 OCTN3 Spermatozoa

Butyryl-L-carnitine 7.32 1.06 ND

g (pH 7.4) in the presence (closed column) or the absence (open column)

8 (12.5 nM) by spermatozoa was measured at 37

each inhibitor. The IC50 value of each compound was estimated from

transport buffer (pH 7.4) in the presence of various concentrations of

murine epididymal spermatozoa. The uptake of [3H]acetyl-L-carnitine

the inhibition of OCTN2- or OCTN3-mediated uptake after subtracting

[3H]carnitine by mouse carnitine/organic cation transporter (OCTN) 2,

OCTN3, and spermatozoa of mice.

Discussion

In this study, characterization and molecular identifica-
tion of transporters involved in the uptake of carnitine

and acetyl carnitine in epididymal spermatozoa were

examined, focusing on OCTN transporters. Although it

was reported that uptake of carnitine in spermatozoa

could be explained by simple diffusion (Jeulin et al.

1994), we demonstrated the presence of a saturable

transport system for carnitine in epididymal spermato-

zoa. This transport activity was reduced by carnitine

analogs and cationic compounds. The apparent dis-

crepancy may reflect species difference (boar and

mouse) and the different uptake times used; Jeulin

et al. (1994) measured at steady state (10 or 20 min)

and in the present study uptake was measured for 3 min.

Table 3 IC50 values of various compounds for the uptake of

[3H]carnitine by mouse carnitine/organic cation transporter (OCTN) 2,

OCTN3, and spermatozoa of mice.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>IC50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OCTN2</td>
</tr>
<tr>
<td>γ-Butyrobetaine</td>
<td>167</td>
</tr>
<tr>
<td>Butyryl-L-carnitine</td>
<td>7.32</td>
</tr>
<tr>
<td>Pyrilamine</td>
<td>41.7</td>
</tr>
<tr>
<td>Quinidine</td>
<td>239</td>
</tr>
<tr>
<td>TEA</td>
<td>132</td>
</tr>
<tr>
<td>Verapamil</td>
<td>58.8</td>
</tr>
</tbody>
</table>

The uptake of l-[3H]carnitine by mouse OCTN2 or OCTN3 expressed

in HEK293 cells or spermatozoa was measured for 3 min at 37 °C in

transport buffer (pH 7.4) in the presence of various concentrations of
each inhibitor. The IC50 value of each compound was estimated from

the inhibition of OCTN2- or OCTN3-mediated uptake after subtracting

the uptake by mock from that by OCTN2- or OCTN3-expressing

HEK293 cells using the MULTI program. ND, not determined.

We observed both Na+-dependent and -independent

transport of carnitine and acetyl carnitine. Eadie–Hofstee

plot analysis indicated a single saturable component with

Km values of 23.6 µM for carnitine and 6.57 µM for

acetylcarnitine in epididymal spermatozoa. Further-

more, expression of the high-affinity carnitine transport-

ers OCTN2 and OCTN3, which are Na+-dependent and

-independent respectively (Tamai et al. 2000), was

confirmed in epididymal spermatozoa. These results

suggested that OCTN2 and OCTN3 are implicated in the

Na+-dependent and -independent transport of both
carnitine and acetyl carnitine, even though kinetic

analysis suggested a single saturable mechanism. This

apparent discrepancy can be explained by the fact that

kinetic analysis could not distinguish between Na+-

dependent and -independent transporters, since the

affinities of these two transporters for carnitine or

acetyl carnitine are not sufficiently different to allow the

separation of two saturable components. The involve-

ment of both OCTN2 and OCTN3 in the transport of
carnitine and acetyl carnitine was further confirmed by

inhibition studies. Concentration-dependent inhibition

studies showed that pyrilamine and γ-butyrobetaine

could be used as selective inhibitors for OCTN2 and

OCTN3 respectively. The IC50 values of pyrilamine and

γ-butyrobetaine for carnitine uptake in epididymal

spermatozoa were 208 and 30.7 µM respectively.

These values are intermediate between the IC50 values

for OCTN2 and OCTN3, supporting the idea that both

OCTN2 and OCTN3 are involved in carnitine uptake in

epididymal spermatozoa.

Interestingly, the expression of OCTN3, which is

localized to the middle piece of the sperm tail, and the

to ratio of OCTN3-positive spermatozoa were increased
during transition though the epididymal tract. Mitochon-

dria, where carnitine is used for fatty acid oxidation,

exist at the middle piece of the sperm tail, and it has been

reported that the conversion of [14C]palmitate to

[14C]CO2 in bovine epididymal spermatozoa is stimu-

lated by addition of carnitine and acetyl carnitine

(Casillas 1972). Acquisition of motility of spermatozoa

occurs during passage through the murine epididymal

tract (Soler et al. 1979, Hinton et al. 1981, Jeulin & Lewin

1996). Accordingly, we suggest that OCTN3 plays a role in fatty acid

oxidation and motility of epididymal spermatozoa by

supplying carnitine/acetyl carnitine to the spermatozoa.

The expression pattern of OCTN2, which is localized to the

principal piece of the sperm tail, is different from that of

OCTN3. Similarly, glucose transporter (GLUT)1 is

localized to the principal piece, and GLUT3 and GLUT5

to the middle piece of the sperm tail (Angulo et al. 1998).

Although the reason for the differential localizations is

not clear, OCTN2 and OCTN3 may have distinct roles in

carnitine/acetyl carnitine transport in epididymal

spermatozoa.
Spermatozoa were usually obtained from whole epididymides. Since cauda spermatozoa are more motile than caput spermatozoa and most of the spermatozoa were reserved in the cauda, it is likely that most of the spermatozoa from whole epididymides were from cauda epididymis. Therefore, we also examined the transport study by spermatozoa from caput, corpus, and cauda epididymides independently. The results suggested that caput and corpus spermatozoa exhibited both Na\(^+\)-dependent and -independent transport of carnitine and acetylcarnitine the same as observed in cauda spermatozoa. Although the ratio of the percentage of OCTN3-positive spermatozoa in corpus was similar to that in cauda, the Na\(^+\)-independent uptake of acetylcarnitine was higher in spermatozoa from the corpus than those in the cauda. This discrepancy may be attributed to other transporters or regional differences of intrinsic carnitine or inorganic ions (Na\(^+\) or K\(^+\), etc.) inside and outside of spermatozoa (Levine & Marsh 1971, Turner et al. 1980, Jeulin & Lewin 1996).

The expression of OCTN1 was very low in epididymal spermatozoa. Since the carnitine transport activity of OCTN1 is very low when compared with that of OCTN2 and OCTN3 (Tamai et al. 2000), OCTN1 might play a role in the transport of compounds other than carnitine, such as ergothionine (Gründemann et al. 2005). Involvement of another carnitine transporter, ATB\(^{0,+}\), which shows low affinity for carnitine (Nakanishi et al. 2001), would be negligible under our experimental conditions, since a substrate of ATB\(^{0,+}\), arginine (5 mM), had no inhibitory effect. However, since the carnitine concentration in epididymal plasma is of millimolar order, involvement of ATB\(^{0,+}\) in physiological carnitine transport by spermatozoa cannot be completely excluded.

The human counterpart of mouse OCTN3 has not yet been identified. However, the human carnitine transporter CT2 (SLC22A16) is selectively expressed in male reproductive tissues, such as Sertoli cells, epididymal epithelial cells, and spermatozoa (Enomoto et al. 2002). Although the amino acid sequence homology between mouse OCTN3 and human CT2 is not high (33%), CT2 may be the physiological functional counterpart of murine OCTN3 in carnitine/acetylcarnitine transport. Mouse SLC22A16 (GenBank Accession Number BC100473) exhibits about 57 and 30% similarity to human CT2 and murine OCTN3 respectively. However, the possible involvement of mouse CT2 in carnitine/acetylcarnitine transport in spermatozoa cannot yet be discussed since the tissue expression profile and transport function of mouse CT2 remain to be established.

This study, the first characterization of carnitine transport in murine spermatozoa, has demonstrated the presence of Na\(^+\)-dependent and -independent transporters, OCTN2 and OCTN3, in epididymal spermatozoa. In addition, it has clarified that both OCTN2 and OCTN3 are expressed in the sperm tail and that the ratio of OCTN3-positive spermatozoa increases during migration though the epididymal tract. Accordingly, these OCTN transporters are likely to play key roles in supplying carnitine and acetylcarnitine to maintain the fertility of spermatozoa.

**Materials and Methods**

**Materials**

L-[Methyl-\(^{3}H\)]carnitine (\[^{3}H\]carnitine, 65 Ci/mmol) was purchased from Amersham Biosciences Corp. Acetyl-L-[N-methyl-\(^{3}H\)]carnitine hydrochloride (\[^{3}H\]acetylcarnitine, 80 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. (St Louis, MO, USA). [Methoxy-\(^{14}C\)]inulin (2.5 mCi/g) and \[^{3}H\]water (1 mCi/g) were purchased from Perkin–Elmer Life Sciences Inc. (Boston, MA, USA). All other reagents, unless otherwise noted, were purchased from Sigma Chemical Co. or Wako Pure Chemical Industries Co. (Osaka, Japan).

**Isolation of epididymal spermatozoa from mice**

Epididymal spermatozoa were isolated from 10-week-old male ddY mice. Whole epididymides were usually dissected out and minced into small fragments on ice in minimum essential medium (Gibco BRL). When regional differences were examined, epididymides were divided into three regions (caput, corpus, and cauda). These fragments were allowed to settle at 37 °C for 5 min, then the supernatant, containing spermatozoa that had swum up, was collected. The collected supernatant was centrifuged (450 g \( \times \) 5 min). The resultant pellet was washed twice with PBS and suspended in transport buffer (125 mM NaCl, 4.6 mM KCl, 5.6 mM (+)-glucose, 1.2 mM CaCl\(_2\), 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 25 mM HEPES (pH 7.4)). Isolated spermatozoa were used for immunofluorescence analysis and transport studies.

**Carnitine transport experiments**

Spermatozoa suspended in transport medium were stored on ice until transport experiments and were used within 3 h of preparation. HEK293 cells expressing mouse OCTN2 or OCTN3 were obtained by transfection of the parental cells with mouse OCTN2/pcDNA3 or mouse OCTN3/pcDNA3 vector respectively (Tamai et al. 2000). The uptake of \[^{3}H\]carnitine and \[^{3}H\]acetylcarnitine by OCTN2- or OCTN3-expressing HEK293 cells or murine spermatozoa was examined by the silicon-layer method, as described previously (Tamai et al. 2000). The cellular protein content was determined according to the method of Bradford using a protein assay kit (Bio-Rad Laboratories) with BSA as the standard (Bradford 1976). In sodium-free experiments, sodium ions were usually replaced with N-methylglucamine, and the cells obtained were suspended in sodium-free transport medium.
Data analysis
The initial uptake rates were usually obtained by measuring the uptake over 3 min for carnitine and acetylcarnitine. The uptake values were usually expressed as the uptake clearance (μL/mg protein/3 min), obtained by dividing the uptake amount in the cells by the concentration of test compound in the medium. [3H]Carnitine or [3H]acetylcarnitine uptake was usually obtained after correction for the extracellularly adsorbed amount, which was estimated from the uptake of [14C]carnitine or [14C]acetyl-carnitine within a short time (about 5 s) at 4 °C. Intracellular water space in epididymal spermatozoa was estimated as the difference between the uptakes of [1H]water and [14C]inulin.

To estimate the kinetic parameters for saturable transport of carnitine or acetylcarnitine, the uptake rate was fitted to the following equation by means of nonlinear regression analysis using the MULTI program (Yamaoka et al. 1981):

\[ V = V_{\text{max}} \cdot C/(K_m + C) + kd \cdot C, \]

where \( V \) and \( C \) are the uptake rate and concentration of carnitine or acetylcarnitine respectively, and \( K_m, V_{\text{max}}, \) and \( kd \) are the half-saturation concentration (Michaelis constant), the maximum transport rate, and the first-order rate constant for non-saturable transport respectively.

The 50% inhibitory concentration (IC(50)) values of various inhibitors for [3H]acetylcarnitine uptake were estimated using the MULTI program according to the following equation:

\[ V = V_0/(1 + (IC_{50})^d) \]

where \( V \) and \( V_0 \) are the uptake rates of [3H]acetylcarnitine in the presence and the absence of inhibitor respectively, and \( d \) is the concentration of inhibitor.

All results for the uptake rates were expressed as mean ± S.E.M. and statistical analysis was performed by ANOVA with Tukey–Kramer’s post hoc test. The criterion of significance was taken to be \( P < 0.05 \).

Immunofluorescence analysis of OCTN1, OCTN2, and OCTN3 in epididymal spermatozoa of mice
Rabbit polyclonal antibodies for mouse OCTN1, OCTN2, and OCTN3 were prepared as described previously (Tamai et al. 2000). Immunofluorescence analysis was done according to our previous reports (Tamai et al. 2001, 2004, Wakayama et al. 2003). Briefly, isolated spermatozoa were fixed on glass slides and incubated with affinity-purified anti-OCTN antibodies or rabbit normal IgG. Then, they were incubated with Alexa Fluor 594 goat anti-rabbit IgG conjugate (Molecular Probes Inc., Eugene, OR, USA). Finally, they were mounted in VECTASHIELD mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) to fix the sample and stain the nuclei. The specimens were examined with an Axiovert S 100 microscope (Carl Zeiss, Jena, Germany) and the images were captured with an AxioCam (Carl Zeiss). OCTN3-positive spermatozoa and head (nuclei) of spermatozoa were counted in a microscope and the ratio of OCTN3-positive spermatozoa (OCTN3-positive cells/head) was determined.

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References


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Levine N & Marsh DJ 1971 Micropuncture studies of the electrolyte transport in individual seminiferous tubules, the epididymis and the vas deferens in rats. Journal of Physiology 213 557–570.


Nakanishi T, Hatanaka T, Huang W, Prasad PD, Leibach FH, Ganapathy ME & Ganapathy V 2001 Na\(^+\)- and Cl\(^-\)-coupled active transport of carnitine by the amino acid transporter ATB\(^{0,3}\) from mouse colon expressed in HRPE cells and Xenopus oocytes. Journal of Physiology 532 297–304.


Wu X, Prasad PD, Leibach FH & Ganapathy V 1998 cDNA sequence, transport function, and genomic organization of human OCTN2, a new member of the organic cation transporter family. Biochemical and Biophysical Research Communications 246 589–595.


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