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

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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, 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.
reported that the Na\(^{+}\)-dependent, high-affinity \(K_m = 4-25 \mu M\) carnitine transporter (Yabuuchi et al. 1999, Tamai et al. 2000, Grundemann et al. 2005). OCTN2 (SLC22A5) is an Na\(^{+}\)-dependent, high-affinity \(K_m = 20 \text{ and } 3 \mu M\) 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\(^{+}\)- and Cl\(^{-}\)-coupled neutral and cationic amino acid transporter ATB\(^{K,+}\) (SLC6A14) can transport carnitine with low affinity \(K_m = 0.83 \text{ mM}\). Furthermore, OCTN2-deficient JVS mice showed male infertility with epididyimal 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 epididyimal 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 epididyimal spermatozoa of mice

The uptakes of \([^3H]\)carnitine and \([^3H]\)acetylcarnitine by epididyimal 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\(^{+}\), but was slightly lower in its absence (extracellular Na\(^{+}\)) 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 \mu L/mg protein), estimated as the difference between the uptakes of \([^3H]\)water and \([^{14}C]\)inulin, in epididyimal spermatozoa, carnitine, and acetylcarnitine seem to be accumulated in the intracellular space of epididyimal 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\(^{+}\) was replaced with lithium or potassium, the uptake of \([^3H]\)acetylcarnitine was higher than that of carnitine into spermatozoa (Fig. 1).

Table 1  Effects of inorganic cations and anions on \([^3H]\)acetylcarnitine uptake by spermatozoa.

<table>
<thead>
<tr>
<th>Cations/anions</th>
<th>Relative uptake (% of control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sodium (control)</td>
<td>100±2</td>
</tr>
<tr>
<td>Lithium</td>
<td>139±3*</td>
</tr>
<tr>
<td>Potassium</td>
<td>117±4*</td>
</tr>
<tr>
<td>N-Methylglucamine</td>
<td>85±4*</td>
</tr>
<tr>
<td>Choline</td>
<td>48±2*</td>
</tr>
<tr>
<td>Anions</td>
<td></td>
</tr>
<tr>
<td>Chloride (control)</td>
<td>100±2</td>
</tr>
<tr>
<td>Thiocyanate</td>
<td>111±2*</td>
</tr>
<tr>
<td>Nitrate</td>
<td>111±2*</td>
</tr>
<tr>
<td>Gluconate</td>
<td>121±3*</td>
</tr>
<tr>
<td>Sulfate</td>
<td>52±2*</td>
</tr>
</tbody>
</table>

The uptake of \([^3H]\)acetylcarnitine (12.5 nM) by spermatozoa was measured for 3 min at 37 °C. When sodium or chloride ions were replaced with other cations or anions, chloride or sodium ions were used as counter-ions respectively. The result is shown as a percentage of the uptake in the presence of sodium chloride. Each value represents the mean ± S.E.M. of four determinations. *Significantly different from the control uptake (sodium chloride buffer) by one-way ANOVA with Tukey–Kramer’s post hoc test (P<0.05).
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 [³H]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 $k_d = 6.55 \pm 1.33 \, \mu L/mg \text{ protein/3 min}$, 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 $k_d = 7.99 \pm 2.63 \, \mu L/mg \text{ protein/3 min}$ (means ± S.E.M.). These results suggested that spermatozoa in mice have a relatively high-affinity carnitine transporter.

Inhibitory effects of several compounds on [³H]acetylcarnitine uptake by murine spermatozoa

To characterize the uptake systems for acetylcarnitine in spermatozoa, we measured [³H]acetylcarnitine uptake in the presence of several compounds. Table 2 shows that 50 μM γ-butyrobetaine and 500 μM 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 [³H]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

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

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

The uptake of [³H]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 ± 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).
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 IC_{50} values of six compounds (\(\gamma\)-butyrobetaine, butyryl-L-carnitine, pyrilamine, quinidine, TEA, and verapamil) were obtained and compared. As shown in Table 3, the IC_{50} values of pyrilamine for OCTN2- or OCTN3-mediated carnitine uptake were 41.7 and 318 \(\mu\)M respectively, suggesting that pyrilamine has a higher affinity for OCTN2. In contrast, \(\gamma\)-butyrobetaine and butyryl-L-carnitine showed a higher affinity for OCTN3 than for OCTN2. The IC_{50} values for OCTN2 and OCTN3 were comparable in the cases of quinidine, TEA, and verapamil. Based on these results, we chose pyrilamine and \(\gamma\)-butyrobetaine as specific (or more strictly, preferential) inhibitors of OCTN2 and OCTN3 respectively. The IC_{50} values of pyrilamine and \(\gamma\)-butyrobetaine for carnitine uptake in epididymal spermatozoa were 208 and 30.7 \(\mu\)M respectively. These results suggest that both OCTN2 and OCTN3 contribute to carnitine uptake in epididymal spermatozoa.
Inhibitors

OCTN2  OCTN3  Spermatozoa

Butyryl-L-carnitine  7.32  1.06  ND
g

of Na
(pH 7.4) in the presence (closed column) or the absence (open column) of Na\(^+\) by spermatozoa was measured at 37\(^\circ\)C in transport buffer (pH 7.4) in the presence (closed column) or the absence (open column) of Na\(^+\). Na\(^+\) was replaced with NMG\(^+\). The results are shown as mean±S.E.M. of four determinations.

Discussion

In this study, characterization and molecular identification of transporters involved in the uptake of carnitine and acetylcarnitine 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 spermatozoa. This transport activity was reduced by carnitine analogs and cationic compounds. The apparent discrepancy 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.

The uptake of \(^{13}\)H[acetyl-L-carnitine by mouse carnitine/organic cation transporter (OCTN) 2, OCTN3, and spermatozoa of mice.

Table 3 IC\(_{50}\) values of various compounds for the uptake of \(^{13}\)H[carnitine by mouse carnitine/organic cation transporter (OCTN) 2, OCTN3, and spermatozoa of mice.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>OCTN2</th>
<th>OCTN3</th>
<th>Spermatozoa</th>
</tr>
</thead>
<tbody>
<tr>
<td>γ-Butyrobetaine</td>
<td>167</td>
<td>1.73</td>
<td>30.7</td>
</tr>
<tr>
<td>Butyryl-L-carnitine</td>
<td>7.32</td>
<td>1.06</td>
<td>ND</td>
</tr>
<tr>
<td>Pyrilamine</td>
<td>41.7</td>
<td>318</td>
<td>208</td>
</tr>
<tr>
<td>Quinidine</td>
<td>239</td>
<td>142</td>
<td>ND</td>
</tr>
<tr>
<td>TEA</td>
<td>132</td>
<td>392</td>
<td>ND</td>
</tr>
<tr>
<td>Verapamil</td>
<td>58.8</td>
<td>20.2</td>
<td>ND</td>
</tr>
</tbody>
</table>

We observed both Na\(^{+}\)-dependent and -independent transport of carnitine and acetylcarnitine. Eadie–Hofstee plot analysis indicated a single saturable component with \(K_m\) values of 23.6 \(\mu\)M for carnitine and 6.57 \(\mu\)M for acetylcarnitine in epididymal spermatozoa. Furthermore, expression of the high-affinity carnitine transporters 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 acetylcarnitine, 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 acetylcarnitine are not sufficiently different to allow the separation of two saturable components. The involvement of both OCTN2 and OCTN3 in the transport of carnitine and acetylcarnitine 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 IC\(_{50}\) values of pyrilamine and γ-butyrobetaine for carnitine uptake in epididymal spermatozoa were 208 and 30.7 \(\mu\)M respectively. These values are intermediate between the IC\(_{50}\) 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 ratio of OCTN3-positive spermatozoa were increased during transition through the epididymal tract. Mitochondria, 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 \(^{14}\)C[palmitate to \(^{14}\)C][CO2 in bovine epididymal spermatozoa is stimulated by addition of carnitine and acetylcarnitine (Casillas 1972). Acquisition of motility of spermatozoa occurs during passage through the murine epididymal tract (Soler et al. 1994), and carnitine is essential for sperm maturation (Casillas & Chaipayungpan 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/acetylcarnitine 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/acetylcarnitine 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. 1977, Jenkins 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 ergothioneine (Grundemann 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/acyetylcarnitine 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). \([\text{Methoxy-}\(^14\)C]\)inulin \((2.5 \text{ mCi/g})\) and \([\text{3H}]\)water \((1 \text{ mCi/g})\) 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 \((450 \times g \times 5 \text{ min})\). The resultant pellet was washed twice with PBS and suspended in transport buffer \((125 \text{ mM NaCl}, 4.8 \text{ mM KCl}, 5.6 \text{ mM } (+)-\)glucose, \(1.2 \text{ mM CaCl}_2, 1.2 \text{ mM KH}_2\text{PO}_4, 1.2 \text{ mM MgSO}_4, 25 \text{ mM HEPES (pH 7.4)})\). 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. \([¹H]Carnitine\) or \([¹H]acetylcarnitine\) uptake was usually obtained after correction for the extracellularly adsorbed amount, which was estimated from the uptake of \([¹H]carnitine\) or \([¹H]acetylcarnitine\) 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 \([¹¹C]inulin\).

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):

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

where \(V\) and \(C\) are the uptake rate and concentration of carnitine or acetylcarnitine respectively, and \(Km, V_{\text{max}},\) 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 \([¹H]\)acetylcarnitine uptake were estimated using the MULTI program according to the following equation:

\[
V = V_0/(1 + (IC_{50}\cdot I))
\]

where \(V\) and \(V_0\) are the uptake rates of \([¹H]\)acetylcarnitine in the presence and the absence of inhibitor respectively, and \(I\) 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 VECTA-SHIELD 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/heads) was determined.

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