REPRODUCTION

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

Daisuke Kobayashi, Ikumi Tamai[1], Yoshimichi Sai, Kazuhiro Yoshida, Tomohiko Wakayama[2], Yasuto Kido, Jun-ichi Nezu[3], Shoichi Iseki[2] 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, [1]Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2641 Yamasaki, Noda, Chiba 278-8510, Japan, [2]Department of Histology and Embryology, Graduate School of Medical Science, Kanazawa University, Takara-machi, Kanazawa 920-8640, Japan and [3]Chugai 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 μM 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.

Reproduction (2007) **134** 651–658

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.*

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^+-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^+- and Cl^--coupled neutral and cationic amino acid transporter $ATB^{0,+}$ (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^+, 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 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^+ was replaced with lithium or potassium, the uptake of [³H]acetylcarnitine
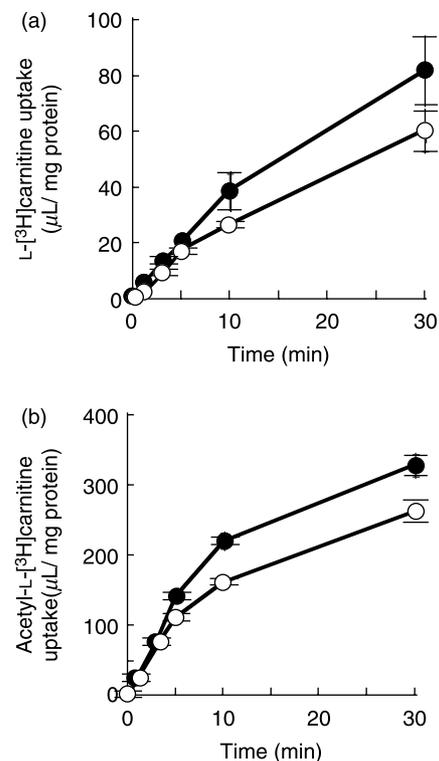
Figure 1 Time course of uptake of (a) [³H]carnitine and (b) [³H]acetyl-carnitine uptake by murine spermatozoa. The uptakes of (a) L-[³H]carnitine (12 nM) and (b) [³H]acetyl-L-carnitine (12.5 nM) by spermatozoa were measured at 37 °C in transport buffer (pH 7.4) in the presence (closed circle) or the absence (open circle) of Na^+. Na^+ was replaced with NMG^+. The results are shown as mean ± s.e.m. of three determinations.

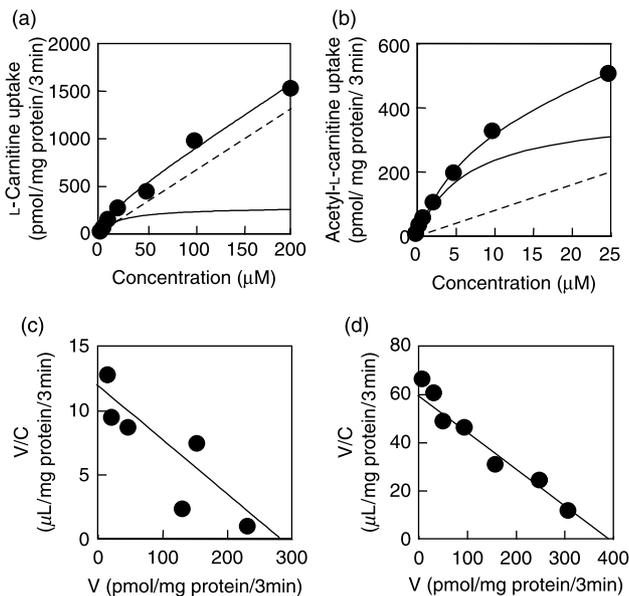**Table 1** Effects of inorganic cations and anions on [³H]acetylcarnitine uptake by spermatozoa.

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

The uptake of [³H]acetyl-L-carnitine (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 [^{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 $K_m = 23.6 \pm 14.5$ μM, $V_{max} = 281 \pm 176$ pmol/mg protein/3 min, and $k_d = 6.55 \pm 1.33$ μL/mg protein/3 min, and those for acetylcarnitine were $K_m = 6.57 \pm 1.77$ μM, $V_{max} = 390 \pm 96$ pmol/mg protein/3 min, and $k_d = 7.99 \pm 2.63$ μL/mg 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 [^{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 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 [^{3}H]acetylcarnitine uptake by spermatozoa. Spermine, spermidine, and arginine were not inhibitory.
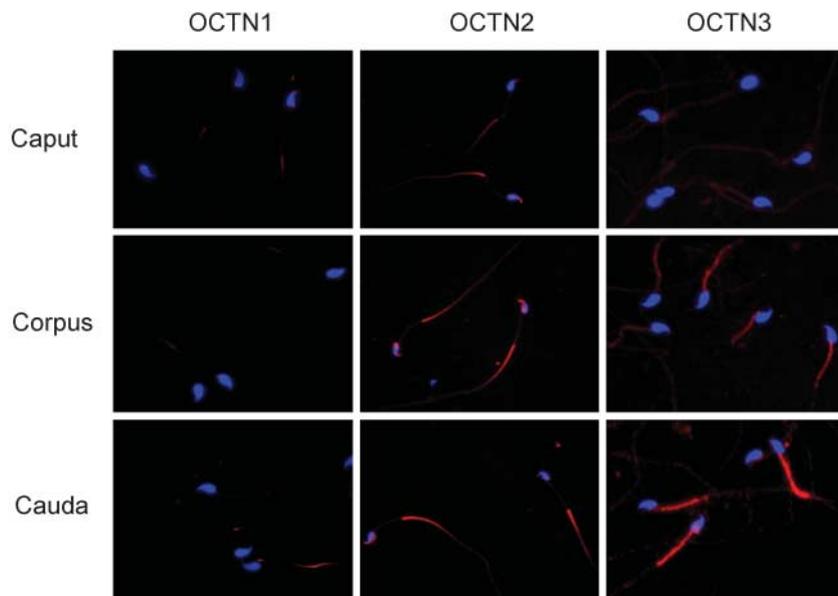
Expression of OCTNs in epididymal spermatozoa of mice

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 epididymidal spermatozoa. The ratio of OCTN3-positive spermatozoa

Figure 2 Concentration dependence of uptake of (a and c) carnitine and (b and d) acetylcarnitine by murine spermatozoa. The uptake of (a) L-carnitine and (b) acetyl-L-carnitine by spermatozoa at various concentrations of carnitine or acety-L-carnitine was measured at 37 °C in transport buffer (pH 7.4) in the presence of Na^+. The amounts of total, saturable, and non-saturable transport, estimated from the kinetics parameters as described in the Results, are represented by the upper solid, lower solid, and dotted lines (a and b). Eadie–Hofstee plots for (c) L-carnitine and (d) acetyl-L-carnitine are shown after subtracting the non-saturable uptake, estimated from the slope of the line through the two points at higher concentration in (a) or (b). Each result represents the mean and s.e.m. (*n* = 3 or 4).

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

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

The uptake of [^{3}H]acetyl-L-carnitine (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).

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.

was significantly higher in corpus ($92.8 \pm 2.2\%$, $n=6$) and cauda ($92.7 \pm 1.9\%$, $n=6$) compared with caput ($71.7 \pm 2.4\%$, $n=6$). 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 (γ-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 cartnitine 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 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 γ-butyrobetaine as specific (or more strictly, preferential) inhibitors of OCTN2 and OCTN3 respectively. The IC_{50} 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.
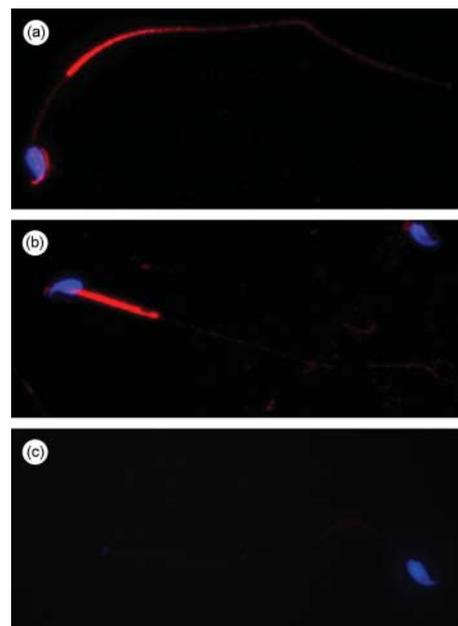
Figure 4 Expressions of OCTN2 and OCTN3 proteins in epididymal spermatozoa in mice. Spermatozoa isolated from cauda epididymis were incubated with affinity-purified antiserum against mouse (a) OCTN2, (b) OCTN3, or (c) normal IgG. The OCTNs appear in red and nuclei in blue.
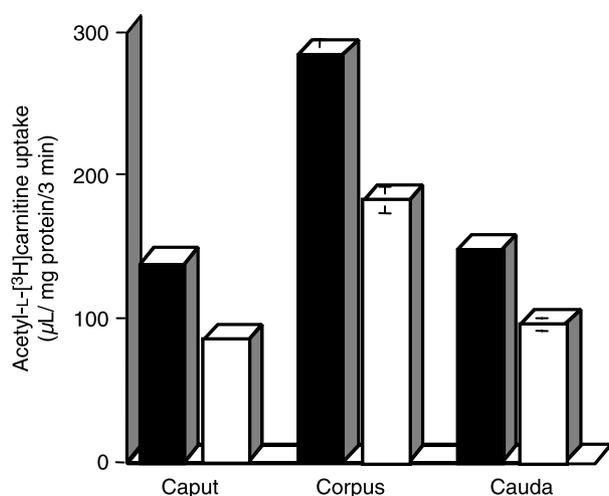
Figure 5 Regional difference of uptake of [³H]acety-L-carnitine by murine epididymal spermatozoa. The uptake of [³H]acetyl-L-carnitine (12.5 nM) by spermatozoa was measured at 37 °C in transport buffer (pH 7.4) in the presence (closed column) or the absence (open column) of Na^+. Na^+ was replaced with 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.

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.

	IC_{50} (µM)		
Inhibitors	OCTN2	OCTN3	Spermatozoa
γ-Butyrobetaine	167	1.73	30.7
Butyryl-L-carnitine	7.32	1.06	ND
Pyrilamine	41.7	318	208
Quinidine	239	142	ND
TEA	132	392	ND
Verapamil	58.8	20.2	ND

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 IC_{50} 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 acetylcarnitine. Eadie–Hofstee plot analysis indicated a single saturable component with K_m values of 23.6 µM for carnitine and 6.57 µM 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 µM 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 though 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 [¹⁴C]palmitate to [¹⁴C]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/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*** × 5 min). The resultant pellet was washed twice with PBS and suspended in transport buffer (125 mM NaCl, 4.8 mM KCl, 5.6 mM (+)-glucose, 1.2 mM $CaCl_2$, 1.2 mM KH_2PO_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. [^{3}H]Carnitine or [^{3}H]acetylcarnitine uptake was usually obtained after correction for the extracellularly adsorbed amount, which was estimated from the uptake of [^{3}H]carnitine or [^{3}H]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 [^{3}H]water and [^{14}C]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):

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

where V and C are the uptake rate and concentration of carnitine or acetylcarnitine respectively, and K_m, V_{max}, and k_d 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 [^{3}H]acetylcarnitine uptake were estimated using the MULTI program according to the following equation:

$$V = V_0/(1 + (I/IC_{50}))$$

where V and V_0 are the uptake rates of [^{3}H]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-postive spermatozoa and head (nuclei) of spermatozoa were counted in a microscope and the ratio of OCTN3-postive spermatozoa (OCTN3-positive cells/head) was determined.

References

Angulo C, Rauch MC, Droppelmann A, Reyes AM, Slebe JC, Delgado-Lopez F, Guaiquil VH, Vera JC & Concha II 1998 Hexose transporter expression and function in mammalian spermatozoa: cellular localization. *Journal of Cellular Biochemistry* **71** 189–203.

Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72** 248–254.

Casillas ER 1972 The distribution of carnitine in male reproductive tissues and its effect on palmitate oxidation by spermatozoal particles. *Biochimica et Biophysica Acta* **280** 545–551.

Casillas ER 1973 Accumulation of carnitine by bovine spermatozoa during maturation in the epididymis. *Journal of Biological Chemistry* **248** 8227–8232.

Casillas ER & Chaipayungpan S 1979 The distribution of carnitine and acetylcarnitine in the rabbit epididymis and the carnitine content of rabbit spermatozoa during maturation. *Journal of Reproduction and Fertility* **56** 439–444.

Casillas ER, Villalobos P & Gonzales R 1984 Distribution of carnitine and acetylcarnitine in the hamster epididymis and in epididymal spermatozoa during maturation. *Journal of Reproduction and Fertility* **72** 197–201.

Cooper TG, Gudermann TW & Yeung CH 1986 Characteristics of the transport of carnitine into the cauda epididymidis of the rat as ascertained by luminal perfusion *in vitro*. *International Journal of Andrology* **9** 348–358.

Dacheux JL & Paquingnon M 1980 Relations between the fertilizing ability, motility and metabolism of epididymal spermatozoa. *Reproduction, Nutrition, Development* **20** 1085–1099.

Deana R, Rigoni F, Francesconi M, Cavallini L, Arslan P & Siliprandi N 1989 Effect of L-carnitine and L-aminocarnitine on calcium transport, motility, and enzyme release from ejaculated bovine spermatozoa. *Biology of Reproduction* **41** 949–955.

Enomoto A, Wempe MF, Tsuchida H, Shin HJ, Cha SH, Anzai N, Goto A, Sakamoto A, Niwa T, Kanai Y et al. 2002 Molecular identification of a novel carnitine transporter specific to human testis. *Journal of Biological Chemistry* **277** 36262–36271.

Grundemann D, Harlfinger S, Golz S, Geerts A, Lazar A, Berkels R, Jung N, Rubbert A & Schomig E 2005 Discovery of the ergothioneine transporter. *PNAS* **102** 5256–5261.

Hinton BT, Snoswell AM & Setchell BP 1979 The concentration of carnitine in the luminal fluid of the testis and epididymis of the rat and some other mammals. *Journal of Reproduction and Fertility* **56** 105–111.

Hinton BT, Brooks DE, Dott HM & Setchell BP 1981 Effects of carnitine and some related compounds on the motility of rat spermatozoa from the caput epididymidis. *Journal of Reproduction and Fertility* **61** 59–64.

Jenkins AD, Lechene CP & Howards SS 1980 Concentrations of seven elements in the intraluminal fluids of the rat seminiferous tubules, rete testis, and epididymis. *Biology of Reproduction* **23** 981–987.

Jeulin C & Lewin LM 1996 Role of free L-carnitine and acetyl-L-carnitine in post-gonadal maturation of mammalian spermatozoa. *Human Reproduction Update* **2** 87–102.

Jeulin C, Soufir JC, Marson J, Paquignon M & Dacheux JL 1987 The distribution of carnitine and acetylcarnitine in the epididymis and epididymal spermatozoa of the boar. *Journal of Reproduction and Fertility* **79** 523–529.

Jeulin C, Dacheux JL & Soufir JC 1994 Uptake and release of free L-carnitine by boar epididymal spermatozoa *in vitro* and subsequent acetylation rate. *Journal of Reproduction and Fertility* **100** 263–271.

Johansen L & Bohmer T 1979 Motility related to the presence of carnitine/acetylcarnitine in human spermatozoa. *International Journal of Andrology* **2** 202–210.

Kobayashi D, Irokawa M, Maeda T, Tsuji A & Tamai I 2005 Carnitine/organic cation transporter OCTN2-mediated transport of carnitine in primary-cultured epididymal epithelial cells. *Reproduction* **130** 931–937.

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.

Marquis NR & Fritz IB 1965 Effects of testosterone on the distribution of carnitine, acetyl carnitine and carnitine acetyltransferase in tissues of the reproductive system of the male rat. *Journal of Biological Chemistry* **240** 2197–2200.

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 $ATB^{0,+}$ from mouse colon expressed in HRPE cells and *Xenopus* oocytes. *Journal of Physiology* **532** 297–304.

Nezu J, Tamai I, Oku A, Ohashi R, Yabuuchi H, Hashimoto N *et al.* 1999 Primary systemic carnitine deficiency is caused by mutations in a gene encoding sodium ion-dependent carnitine transporter . *Nature Genetics* **21** 91–94.

Ohashi R, Tamai I, Yabuuchi H, Nezu J, Oku A, Sai Y, Shimane M & Tsuji A 1999 Na^+-dependent carnitine transport by organic cation transporter (OCTN2): its pharmacological and toxicological relevance. *Journal of Pharmacology and Experimental Therapeutics* **291** 778–784.

Radigue C, Es-Slami S & Soufir JC 1996 Relationship of carnitine transport across the epididymis to blood carnitine and androgens in rats. *Archives of Andrology* **37** 27–31.

Sekine T, Kusuhara H, Utsunomiya-Tate N, Tsuda M, Sugiyama Y, Kanai Y & Endou H 1998 Molecular cloning and characterization of high-affinity carnitine transporter from rat intestine. *Biochemical and Biophysical Research Communications* **251** 586–591.

Soler C, Yeung CH & Cooper TG 1994 Development of sperm motility patterns in the murine epididymis. *International Journal of Andrology* **17** 271–278.

Tamai I, Yabuuchi H, Nezu J, Sai Y, Oku A, Shimane M & Tsuji A 1997 Cloning and characterization of a novel human pH-dependent organic cation transporter. *FEBS Letters* **419** 107–111.

Tamai I, Ohashi R, Nezu J, Yabuuchi H, Oku A, Shimane M, Sai Y & Tsuji A 1998 Molecular and functional identification of sodium ion-dependent, high affinity human carnitine transporter OCTN2. *Journal of Biological Chemistry* **273** 20378–20382.

Tamai I, Ohashi R, Nezu J, Sai Y, Kobayashi D, Oku A, Shimane M & Tsuji A 2000 Molecular and functional characterization of organic cation/carnitine transporter family in mice. *Journal of Biological Chemistry* **275** 40064–40072.

Tamai I, China K, Sai Y, Kobayashi D, Nezu J, Kawahara E & Tsuji A 2001 Na^+-coupled transport of L-carnitine via high-affinity carnitine transporter OCTN2 and its subcellular localization in kidney. *Biochimica et Biophysica Acta* **1512** 273–284.

Tamai I, Nakanishi T, Kobayashi D, China K, Kosugi Y, Nezu J, Sai Y & Tsuji A 2004 Functional characterization and renal apical membrane localization of organic cation transporter OCTN1 (SLC22A4). *Molecular Pharmaceutics* **1** 57–66.

Toshimori K, Kuwajima M, Yoshinaga K, Wakayama T & Shima K 1999 Dysfunctions of epididymis as a result of primary carnitine deficiency in juvenile visceral steatosis mice. *FEBS Letters* **446** 323–326.

Turner TT, Hartmann PK & Howards SS 1977 *In vivo* sodium, and sperm concentrations in the rat epididymis. *Fertility and Sterility* **28** 191–194.

Wakayama T, Koami H, Ariga H, Kobayashi D, Sai Y, Tsuji A, Yamamoto M & Iseki S 2003 Expression and functional characterization of the adhesion molecule spermatogenic immunoglobulin superfamily in the mouse testis. *Biology of Reproduction* **68** 1755–1763.

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.

Wu X, Huang W, Prasad PD, Seth P, Rajan DP, Leibach FH, Chen J, Conway SJ & Ganapathy V 1999 Functional characteristics and tissue distribution pattern of organic cation transporter 2 (OCTN2), an organic cation/carnitine transporter. *Journal of Pharmacology and Experimental Therapeutics* **290** 1482–1492.

Wu X, George RL, Huang W, Wang H, Conway SJ, Leibach FH & Ganapathy V 2000 Structural and functional characteristics and tissue distribution pattern of rat OCTN1, an organic cation transporter, cloned from placenta. *Biochimica et Biophysica Acta* **1466** 315–327.

Xuan W, Lamhonwah AM, Librach C, Jarvi K & Tein I 2003 Characterization of organic cation/carnitine transporter family in human sperm. *Biochemical and Biophysical Research Communications* **306** 121–128.

Yabuuchi H, Tamai I, Nezu J, Sakamoto K, Oku A, Shimane M, Sai Y & Tsuji A 1999 Novel membrane transporter OCTN1 mediates multispecific, bi-directional, and pH-dependent transport of organic cations. *Journal of Pharmacology and Experimental Therapeutics* **289** 768–773.

Yamaoka K, Tanigawara Y, Nakagawa T & Uno T 1981 A pharmacokinetic analysis program (MULTI) for microcomputer. *Journal of Pharmacobio-Dynamics* **4** 879–885.

Yeung CH, Cooper TG & Waites GMH 1980 Carnitine transport into the perfused epididymis of the rat: regional differences, stereospecificity, stimulation by choline, and the effect of other luminal factors. *Biology of Reproduction* **23** 294–304.