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Transport of butyryl-L-carnitine, a potential prodrug, via the carnitine transporter OCTN2 and the amino acid transporter ATB$^{0,+}$

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Abstract

L-Carnitine is absorbed in the intestinal tract via the carnitine transporter OCTN2 and the amino acid transporter ATB$^{0,+}$. Loss-of-function mutations in OCTN2 may be associated with inflammatory bowel disease (IBD), suggesting a role for carnitine in intestinal/colonic health. In contrast, ATB$^{0,+}$ is upregulated in bowel inflammation. Butyrate, a bacterial fermentation product, is beneficial for prevention/treatment of ulcerative colitis. Butyryl-L-carnitine (BC), a butyrate ester of carnitine, may have potential for treatment of gut inflammation, since BC would supply both butyrate and carnitine. We examined the transport of BC via ATB$^{0,+}$ to determine if this transporter could serve as a delivery system for BC. We also examined the transport of BC via OCTN2. Studies were done with cloned ATB$^{0,+}$ and OCTN2 in heterologous expression systems. BC inhibited ATB$^{0,+}$-mediated glycine transport in mammalian cells (IC_{50}, 4.6 ± 0.7 mM). In *Xenopus laevis* oocytes expressing human ATB$^{0,+}$, BC induced Na^+-dependent inward currents under voltage-clamp conditions. The currents were saturable with a $K_{0.5}$ of 1.4 ± 0.1 mM. Na^+ activation kinetics of BC-induced currents suggested involvement of two Na^+ per transport cycle. BC also inhibited OCTN2-mediated carnitine uptake (IC_{50}, 1.5 ± 0.3 μM). Transport of BC via OCTN2 is electrogenic, as evidenced from BC-induced inward currents. These currents were Na^+ dependent and saturable ($K_{0.5}$, 0.40 ± 0.02 μM). We conclude that ATB$^{0,+}$ is a low-affinity/high-capacity transporter for BC, whereas OCTN2 is a high-affinity/low-capacity transporter. ATB$^{0,+}$ may mediate intestinal absorption of BC when OCTN2 is defective.

Keywords

butyryl-L-carnitine; OCTN2; ATB^0; $^+$; carnitine; butyrate; Crohn's disease; necrotizing enterocolitis

Carnitine (β-hydroxy γ-trimethylaminobutyrate) is obligatory for transport of long-chain fatty acids into mitochondria for subsequent β-oxidation (1); it plays a critical role in energy metabolism of the tissues that derive substantial portion of their metabolic energy from fatty acid oxidation such as heart, skeletal muscle, liver, and placenta (40, 41, 49). The biological importance of carnitine is underscored by the severe clinical consequences of carnitine deficiency as seen in humans (27, 61). Two distinct types of carnitine deficiency states have

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been identified: primary carnitine deficiency, which arises from defects in the plasma membrane carnitine transporter, and secondary carnitine deficiency, which arises from defects in any of the enzymes involved in mitochondrial β-oxidation. Patients with primary carnitine deficiency excrete carnitine in urine because of defective renal reabsorption, and plasma and tissue levels of carnitine drop below 10% of normal values in these patients (6, 47, 50, 59, 60). Patients with secondary carnitine deficiency accumulate organic acids, which enhance urinary excretion of carnitine in the form of acyl-carnitines (41, 42).

The role of carnitine in the gastrointestinal tract has become a topic of interest recently because of epidemiological studies linking mutations in genes coding for plasma membrane transporters *OCTN1* (*SLC22A4*) and *OCTN2* (*SLC22A5*) with Crohn's disease: a missense substitution 1672C→T in *OCTN1*causing amino acid substitution L503F and a G→C transversion in the promoter region of *OCTN2* (−207 G→C) disrupting a heat shock binding element. Both of these mutations are in strong linkage disequilibrium and create a two-allele risk haplotype, which increases the odds of acquiring Crohn's disease 1.5- to 12-fold (56). These mutations have been shown to decrease the transport function of OCTN1 and reduce the expression of OCTN2, thus potentially causing tissue carnitine deficiency (38). Findings from these genetic studies have now been replicated in over 19 studies in ethnically diverse populations (13, 14, 17, 29, 30, 35–38, 45, 53–55, 58, 65). These association studies suggest that carnitine transport deficiency might play a role in the pathogenesis of Crohn's disease. However, it has to be pointed out that, while the role of OCTN2 in carnitine uptake in mammalian tissues is unequivocal, the role of OCTN1 in carnitine uptake is controversial. Some studies have demonstrated carnitine uptake via the transporter, whereas other studies were unable to confirm this function.

Carnitine is transported in the intestine by OCTN2, and the process is developmentally regulated (10, 11, 15, 25), thus making carnitine and fatty acid oxidation highly relevant in normal gut function. There are several reports linking inhibition of fatty acid oxidation in the gut with inflammation (23, 43, 44); likewise, L-carnitine has also been shown to play a protective role in gut inflammation (7, 18). Another transporter that might play a role in the intestinal absorption of carnitine is $ATB^{0,+}$. This is an amino acid transporter that also functions as a low-affinity/high-capacity transporter for carnitine (22, 34). Interestingly, although the expression of OCTN2 is reduced in patients with inflammatory bowel disease (IBD), the expression of $ATB^{0,+}$ is markedly upregulated in this disease (8, 51).

Butyrate, a short-chain fatty acid, is produced in the large bowel by bacterial fermentation of dietary fiber and plays an important role in the maintenance of colonic health (32, 46). Butyrate has been shown to be transported in the epithelial cells lining the intestine and colon by a specific Na^+-coupled transporter known as SMCT1 (SLC5A8; see Refs. 20 and 33) and via the Na ±-independent transporter MCT1 (19). This bacterial metabolite reduces production of proinflammatory cytokines (5), decreases colonic paracellular permeability (26), and suppresses cyclooxygenase-2 activation (52). These effects are critical in the prevention of gut inflammation. Recently, butyrate has been shown to play a significant role in tumor suppression (21). Butyrate is also a ligand for certain specific G-protein-coupled receptors (GPR41 and GPR43) that are found on immune cells (2, 28). This may have relevance to the potential immunosuppressive function of butyrate in the intestinal tract. GPR41 and GPR43 are known to modulate gut motility (9, 24) and leptin production (3, 64).

Thus available evidence supports an important role for carnitine and butyrate in the maintenance of intestinal/colonic health and prevention of gut inflammation. However, because the expression of OCTN2, the high-affinity transporter for carnitine, is reduced in IBD, the resultant carnitine deficiency in the intestinal and colonic epithelial cells may contribute to the inflammatory process. There is no information in the literature on the

expression of SLC5A8 during gut inflammation; therefore, it is not known whether butyrate uptake in epithelial cells is compromised during inflammation. Because $ATB^{0,+}$ is upregulated in IBD, we asked whether this transporter can be exploited as a delivery system for butyryl-L-carnitine (BC), a potential prodrug capable of providing the cells with carnitine and butyrate.

MATERIALS AND METHODS

Synthesis of BC

BC ($C_{11}H_{22}ClNO_4$) was synthesized by Custom Synthesis Services. Butyric acid (60 ml) and butyryl chloride (59.4 mmol) were warmed at 80°C for 3 h under nitrogen. This mixture was added to L-carnitine chloride (55.2 mmol), and the warming was continued at 80°C for another 1 h. The majority of the solvent was removed by roto-vap at 60°C under high vacuum. The residue was dissolved in 25 ml of isopropyl alcohol and then added to 200 ml of acetone with 100 ml of ethyl ether. Upon standing, crystals began to form in this solution. The crystals were isolated by vacuum filtration and washed with acetone. The concentration of the filtrate provided two additional crops of identically pure material. The combined crops of BC were thoroughly dried under high vacuum for several days before final analysis. The molecular identity of the product as BC was confirmed by electro-spray ionization mass spectrometry and elemental analysis (C, H, N, Cl) by nuclear magnetic resonance spectroscopy (^{1}HNMR-^{13}C NMR). BC is a zwitterion with a molecular weight 232.2 (Fig. 1). The synthesized compound was stored at −20°C.

Molecular cloning of human OCTN2 and $ATB^{0,+}$

OCTN2 was previously cloned by us from a human placental trophoblast cell line (JAR) cDNA library (NM_003060; see Ref. 63), and $ATB^{0,+}$ was cloned from a mammary tumor cell line (MCF-7) cDNA library (AF151978; see Ref. 34) using standard techniques as previously described.

Functional expression of $ATB^{0,+}$ and OCTN2 in human retinal pigment epithelial cells

The human $ATB^{0,+}$ cDNA and the human OCTN2 cDNA were used for functional expression in human retinal pigment epithelial (HRPE) cells in the analysis of their role in the transport of BC. The vaccinia virus expression system was used for this purpose. This procedure involves infection of the cells with a recombinant vaccinia virus carrying the gene for T7 RNA polymerase, followed by lipofectin-mediated transfection of the cells with plasmid DNA in which the cDNA insert is under the control of T7 promoter. Glycine (40 μM radiolabeled glycine; 31.6 mM unlabeled glycine) was used as the substrate for $ATB^{0,+}$, and carnitine (30 nM; only radiolabeled carnitine without any addition of unlabeled carnitine) was used as the substrate for OCTN2. Transport in cDNA-transfected cells was measured at 37°C for 30 min. Transport in cells transfected with vector alone was also measured in parallel to account for constitutive transport activity that was present in HRPE cells. This constitutive activity was subtracted for transport activity measured in cDNA-transfected cells to calculate cDNA-specific activity. The transport of glycine and carnitine was linear under these conditions. The transport buffer was 25 mM HEPES/Tris (pH 7.5) containing 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose. Interaction of L-carnitine and BC with the transporters was assessed by monitoring the ability of these compounds to inhibit $ATB^{0,+}$-mediated glycine transport or OCTN2-mediated carnitine transport. The influence of BC on the kinetic parameters of $ATB^{0,+}$ and OCTN2 was investigated by analyzing the saturation kinetics of glycine transport ($ATB^{0,+}$) and carnitine transport (OCTN2) in the absence and presence of BC. The competitive nature of the inhibition of $ATB^{0,+}$-mediated glycine uptake by BC was confirmed by two different approaches: analysis of saturation kinetics of glycine uptake in the absence and presence of

a fixed concentration of BC and analysis of glycine uptake at two different concentrations in the presence of increasing concentrations of BC.

Functional expression of OCTN2 and $ATB^{0,+}$ in Xenopus laevis oocytes

Capped cRNAs from the cloned human OCTN2 cDNA and human $ATB^{0,+}$ cDNA were synthesized using the mMESSAGE mMACHINE kit (Ambion, Austin, TX). Mature oocytes (stage IV or V) from *Xenopus laevis* were isolated by treatment with collagenase A (1.6 mg/ml), manually defolliculated, and maintained at 18°C in modified Barth's medium, supplemented with 10 mg/ml gentamycin as described previously (12). On the following day, oocytes were injected with 50 ng of cRNA. Water-injected oocytes served as controls. The oocytes were used for electrophysiological studies 6 days after cRNA injection. Electrophysiological studies were performed by the two-microelectrode voltage-clamp method (12). Oocytes were superfused with an NaCl-containing buffer (in mM: 100 NaCl, 2 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 3 HEPES, 3 2-(*N*-morpholino)-ethanesulfonic acid, and 3 Tris, pH 7.5), followed by the same buffer containing carnitine or BC. The membrane potential was clamped at −50 mV. The differences between the steady-state currents measured in the presence and absence of substrates were considered as the substrate-induced currents. In the analysis of the saturation kinetics of substrate-induced currents, the kinetic parameter $K_{0.5}$ (i.e., the substrate concentration necessary for the induction of half-maximal current) was calculated by fitting the values of the substrate-induced currents to the Michaelis-Menten equation.

The dependence of $ATB^{0,+}$-mediated transport of BC on Na^+ was determined by comparing the BC-induced currents in the presence of Na^+ and in the absence of Na^+ [*N*-methyl-D-glucamine chloride replacing NaCl isoosmotically]. The Na^+-activation kinetics was analyzed by measuring the substrate-specific currents in the presence of increasing concentrations of Na^+ (the concentration of Cl^- kept constant at 100 mM). In these experiments, the composition of the superfusion buffer was modified to contain 2 mM potassium gluconate, 1 mM $MgSO_4$, and 1 mM calcium gluconate in place of KCl, $MgCl_2$, and $CaCl_2$, respectively. The data from these experiments were analyzed by the Hill equation to determine the $K_{0.5}$ value for Na^+ (i.e., the concentration of Na^+ necessary for half-maximal activation) and the Hill coefficient (h; the number of Na^+ ions involved in the activation process). The kinetic parameters were determined using the commercially available computer program Sigma Plot, version 10.0 (SPSS Science, Chicago, IL).

Data analysis

Experiments with HRPE cells were repeated three times with three independent transfections, and transport measurements were made in duplicate in each experiment. Electrophysiological measurements of substrate-induced currents were repeated at least three times with separate oocytes. The data are presented as means ± SE of these replicates. Statistical analysis was done by Student's t-test, and a $P < 0.05$ was considered significant. Data for saturation kinetics were analyzed according to the Michaelis-Menten equation

$$y = a \times x/(b+x)$$

where y is uptake rate or substrate-induced current, a is maximal transport velocity (V_{max}) or maximal substrate-induced current (I_{max}), b is the Michaelis constant (K_t in uptake measurement studies or $K_{0.5}$ in current measurement studies), and x is substrate concentration. Data for Na^+ activation kinetics were analyzed according to the Hill equation

$$I = I_{max} \times x^h / (K_{0.5}^h + x^h)$$

where I is substrate-induced current, I_{max} is current induced by a maximal concentration of Na^+, x is concentration of Na^+, $K_{0.5}$ is the concentration of Na^+ necessary for induction of half-maximal current, and h is the Hill coefficient. The IC_{50} (i.e., the concentration of inhibitor needed to cause 50% inhibition of uptake) values were calculated by fitting the data from the dose-response experiments to the equation

$$y = y^0 + a \times b / (b + x)$$

where y is uptake in the presence of the inhibitor, y^0 is uptake in the presence of a maximal concentration of the inhibitor, a is uptake in the absence of the inhibitor, b is concentration of the inhibitor needed for 50% inhibition, and x is concentration of the inhibitor.

RESULTS

Transport of BC via human $ATB^{0,+}$

We first examined the interaction of BC with human $ATB^{0,+}$ expressed heterologously in HRPE cells by assessing the effect of BC on $ATB^{0,+}$-mediated glycine transport. Transport of glycine in cDNA-transfected cells was ~30 times greater than in vector-transfected cells, indicating robust expression of human $ATB^{0,+}$ cDNA. The cDNA-specific glycine transport was inhibited by BC in a dose-dependent manner (Fig. 2). The IC_{50} value for this inhibition was 4.6 ± 0.7 mM. In parallel, we also compared the effect of carnitine on cDNA-specific glycine transport. Carnitine inhibited glycine transport more potently than BC. The IC_{50} value for inhibition by carnitine was 0.7 ± 0.1 mM. Kinetic analysis revealed that the inhibition of $ATB^{0,+}$-mediated glycine transport by BC was associated with an increase in the Michaelis constant for glycine with relatively less effect on maximal velocity (Fig. 3*A*). The values for K_m and V_{max} for glycine uptake in the absence of BC were 0.32 ± 0.08 mM and 1,066 ± 94 pmol · 10^{-6} cells · min^{-1}, respectively. The corresponding values in the presence of 2 mM BC were 0.45 ± 0.06 mM (40% increase compared with control value) and 887 ± 44 pmol · 10^{-6} cells · min^{-1} (17% decrease compared with control value). These data suggest that the inhibition of $ATB^{0,+}$-mediated glycine uptake by BC is likely to be competitive. This was confirmed by a Dixon plot (Fig. 3*B*). The inhibition constant (K_i) calculated from the Dixon plot was 2.5 mM.

These studies with the mammalian cell expression system show that BC interacts with human $ATB^{0,+}$ as evident from the inhibition of cDNA-mediated glycine transport, but they do not show whether or not BC is actually transported via $ATB^{0,+}$. To determine whether BC is a transportable substrate for this transporter, we employed the *X. laevis* oocyte expression system. In this system, the transport of any given compound into oocytes can be monitored by inward currents induced by the compound under voltage-clamp conditions. This is possible because $ATB^{0,+}$ is an electrogenic transporter and transport of its substrates is associated with membrane depolarization. Superfusion of $ATB^{0,+}$-expressing oocytes led to marked inward currents (Fig. 4). These currents were not detectable in the absence of Na^+, indicating that the presence of Na^+ is obligatory for the induction of the currents. There were little BC-inducible currents in water-injected oocytes even in the presence of Na^+, showing that the observed currents were specific because of transport via $ATB^{0,+}$. We could not perform Cl^- dependence studies because BC was prepared as a chloride salt; therefore, studies in the absence of Cl^- were not feasible. BC-induced currents in the presence of Na^+

were saturable with a Michaelis constant of 1.5 ± 0.1 mM (Fig. 5*A*). Na^+ activation kinetics of BC-induced currents in $ATB^{0,+}$-expressing oocytes showed a sigmoidal relationship, indicating participation of multiple Na^+ in the activation process (Fig. 5*B*). The Hill coefficient (*h*) for the activation process was 1.8 ± 0.1. Thus the Na^+-BC stoichiometry for transport via $ATB^{0,+}$ is 2:1.

Transport of BC via human OCTN2

OCTN2 is an Na^+-coupled transporter for carnitine. It can also transport carnitine esters with the short-chain fatty acids acetate and propionate (62). Carnitine esters with longer-chain fatty acids are known to interact with the transporter (48), but it has not been demonstrated whether or not these esters are actually transportable substrates for OCTN2. Therefore, we wondered whether BC is a transportable substrate for this transporter. Because we studied the transport of BC via $ATB^{0,+}$, we first examined the interaction of BC with human OCTN2 by monitoring the effect of BC on OCTN2-mediated carnitine transport in HRPE cells following heterologous expression of OCTN2 cDNA. These studies showed that BC inhibited OCTN2-specific carnitine transport very effectively (Fig. 6). The IC_{50} value for this inhibition was 1.5 ± 0.3 μM. Unlabeled carnitine competed with [^{3}H]carnitine for transport via OCTN2. The IC_{50} value for this interaction was 2.1 ± 0.5 μM. The inhibition of carnitine transport by BC was competitive, with the process being associated primarily with an increase in the Michaelis constant for carnitine without any significant change in maximal velocity of transport (data not shown).

Again, these data with the mammalian cell expression system demonstrate that BC interacts with OCTN2 but do not show whether or not BC is actually transported via the transporter. Therefore, we used the *X. laevis* oocyte expression system. It has already been shown that OCTN2 is electrogenic and that exposure of OCTN2-expressing oocytes to carnitine induces inward currents under voltage-clamp conditions (57). Using this expression system, we could show that exposure of OCTN2-expressing oocytes to BC induced inward currents. These currents were obligatorily dependent on Na^+ (Fig. 7*A*). Such currents were not detectable in water-injected oocytes, demonstrating that the observed currents are specific for OCTN2. The current-voltage relationship showed that membrane potential plays a critical role in the energization of the transporter. Hyperpolarization increased the magnitude of BC-induced currents, whereas depolarization decreased the magnitude of the currents (Fig. 7*B*). Similar results were obtained with carnitine, although the carnitine-induced currents were significantly smaller than those induced by BC. Saturation kinetics of BC-induced currents in OCTN2-expressing oocytes showed that the transporter has high affinity for BC. The Michaelis constant for the transport process was 0.40 ± 0.02 μM (Fig. 8).

DISCUSSION

Our data in this report demonstrate that BC, a potential prodrug supplying both carnitine and butyrate, is a transportable substrate for the amino acid transporter $ATB^{0,+}$. Our previous studies have shown that $ATB^{0,+}$ is able to transport carnitine and propionyl-L-carnitine (34). Even though BC is similar to propionyl-L-carnitine, BC has unique therapeutic potential in the treatment of IBD. Butyrate is a well known suppressant of gut inflammation. Furthermore, the expression of $ATB^{0,+}$ is increased severalfold in humans with colonic inflammation. Thus $ATB^{0,+}$ offers a unique means to deliver antiinflammatory drugs in intestinal/colonic epithelial cells. Our findings that BC is a substrate for $ATB^{0,+}$ suggest that this prodrug can enter the intestinal/colonic epithelial cells very effectively via the transporter under inflammatory conditions. BC is also a prodrug of carnitine. Mutations in OCTN2 leading to decreased carnitine transport function are associated with IBD, implying that carnitine deficiency in intestinal/colonic epithelial cells may contribute to gut pathology in IBD. BC may be able to provide carnitine to intestinal/colonic epithelial cells in IBD via

$ATB^{0,+}$. The transport of BC via $ATB^{0,+}$ has many desirable features for the delivery of butyrate and carnitine in intestinal/colonic epithelial cells under conditions of inflammation. First, the transporter is upregulated in IBD, making it a more effective mode of drug delivery, specifically in inflamed cells. Second, the transporter is highly energetic, being driven by transmembrane gradients of Na^+ and Cl^-, which enables concentrative entry of BC in inflamed cells. Third, $ATB^{0,+}$ functions as a low-affinity/high-capacity transporter for BC. Therefore, sufficient quantities of butyrate and carnitine can be supplied to the epithelial cells via this transporter.

Our studies also show that BC is transported via OCTN2. The transport process is of the high-affinity/low-capacity type. This transporter is therefore not suitable for delivery of high levels of carnitine or butyrate in cells. Although this is not a desirable feature in the intestinal tract, high-affinity delivery of BC in systemic organs such as cardiac muscle, skeletal muscle, and kidney may have advantages because plasma levels of BC may not reach high levels following oral dosing of this compound.

The role of fatty acid oxidation in the gut is underscored by the fact that inhibition of gut fatty acid β-oxidation by specific inhibitors (e.g., sodium 2-bromo-octanoate) has been shown to produce experimental colitis (43, 44), highlighting the crucial role of fatty acid oxidation in this tissue. Our studies in the *juvenile visceral steatosis* (*jvs*) mouse, which has a defective OCTN2 transporter, have shown a clear phenotype in the gut (66). The homozygous *jvs* mouse develops villous structure atrophy, inflammation, spontaneous perforations, and pus formation in the peritoneal cavity (49a). Furthermore, several anecdotal reports have shown a beneficial effect of carnitine and butyrate enemas in some cases of distal IBD (4, 16, 18, 39). Short-chain fatty acids not only exert a nutritional effect on the gut but also are protective for enterocytes. In addition, they activate transcription of genes coding for antiinflammatory mediators (4, 31). Thus the intestinal transport of BC via $ATB^{0,+}$ is likely to be beneficial to patients with IBD and also in patients with genetic defects in OCTN2.

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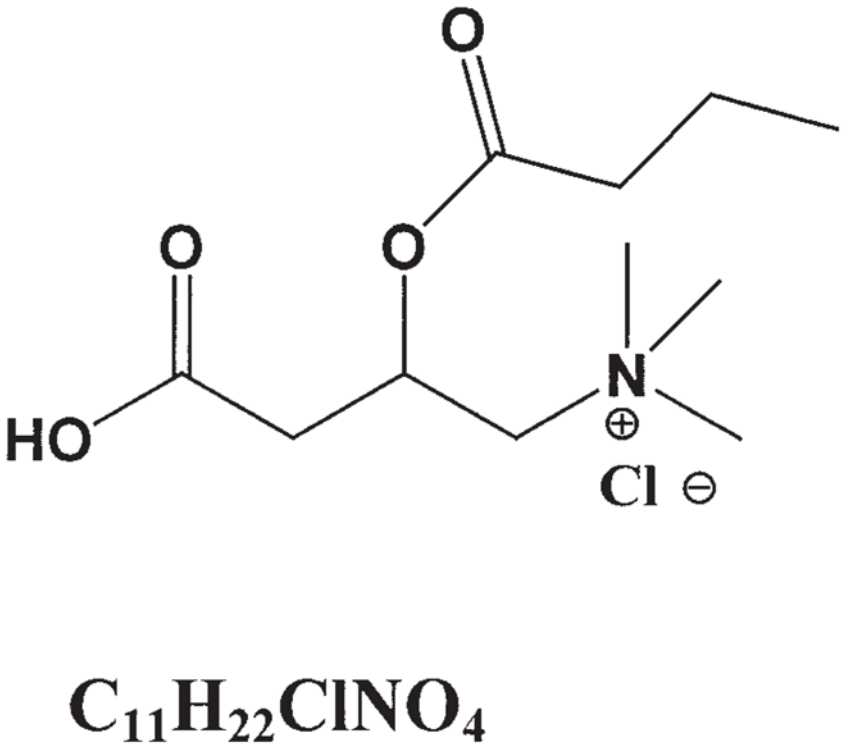

Fig 1.
Molecular structure of butyryl-L-carnitine.

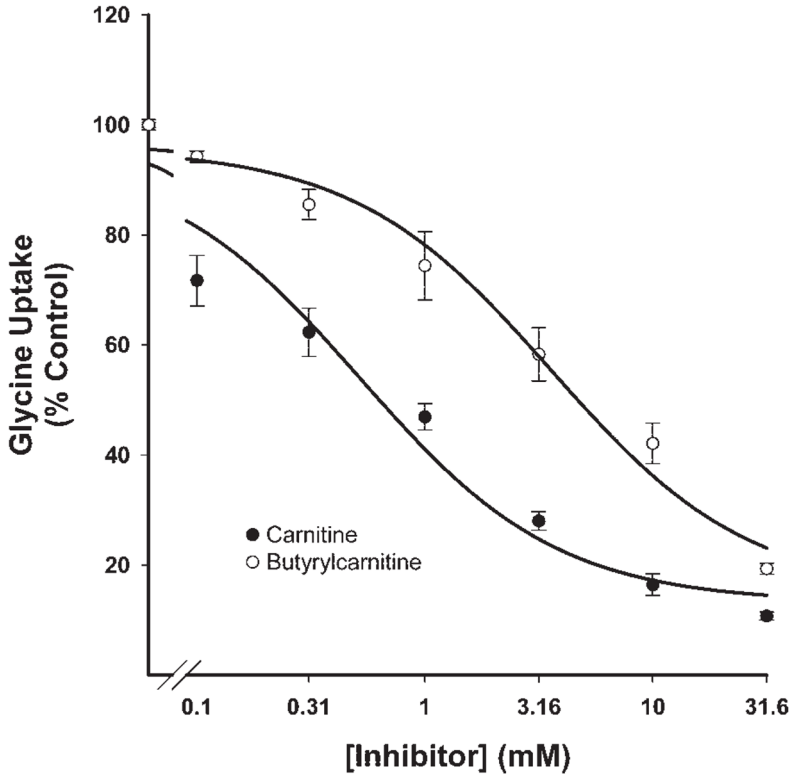

Fig 2.

Inhibition of human (h) $ATB^{0,+}$-mediated glycine transport by L-carnitine and butyryl-L-carnitine. Human retinal pigment epithelial (HRPE) cells were transfected with either vector alone or $hATB^{0,+}$ cDNA. Uptake of [^{3}H]glycine (40 μM) was measured in the presence of increasing concentrations of L-carnitine (●) and butyryl-L-carnitine (○) over a concentration range of 0–31.6 mM. Uptake in vector-transfected cells was subtracted from uptake in cDNA-transfected cells to calculate $hATB^{0,+}$-specific transport. Transport in the absence of inhibitors was taken as 100%.

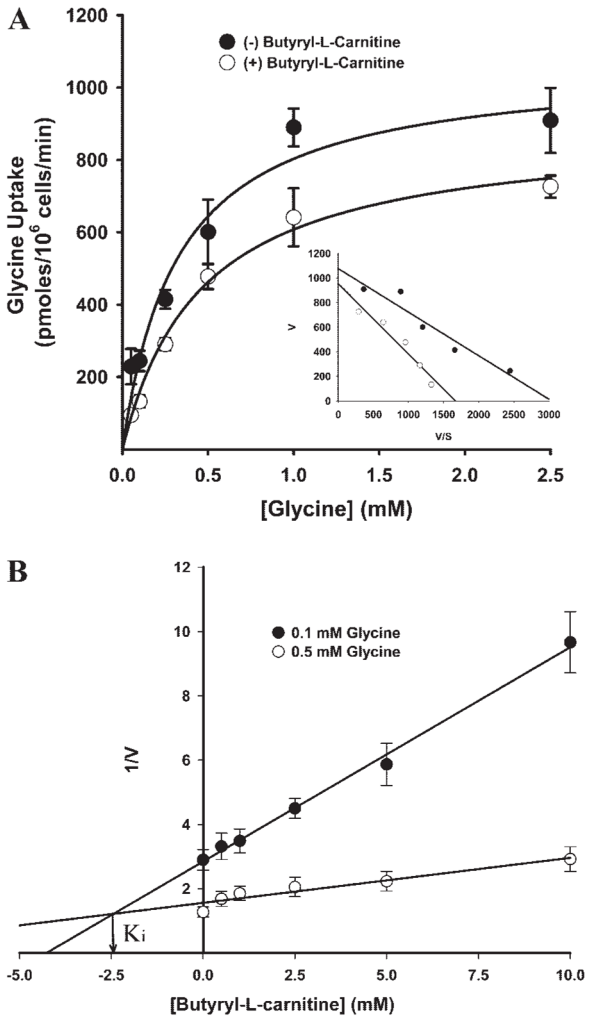

Fig 3.

Competitive inhibition of glycine uptake by butyryl-L-carnitine in hATB$^{0,+}$-expressing HRPE cells. *A*: saturation kinetics of hATB$^{0,+}$-mediated glycine uptake in the absence (●) and presence (○) of 2 mM butyryl-L-carnitine was analyzed in hATB$^{0,+}$ -expressing HRPE cells. *Inset*: Eadie-Hofstee plots (V/S vs. V, where V is glycine uptake in pmol · 10^{-6} cells · min^{-1} and S is glycine concentration in mM). *B*: uptake of glycine was measured at two different concentrations (0.1 and 0.5 mM) in the presence of increasing concentrations of butyryl-L-carnitine (concentration range, 0–10 mM) in hATB$^{0,+}$-expressing HRPE cells. Data are presented as Dixon plots (butyryl-L-carnitine concentration vs. 1/V). The intersect of the two lines gives an estimate of the inhibition constant (K_i).

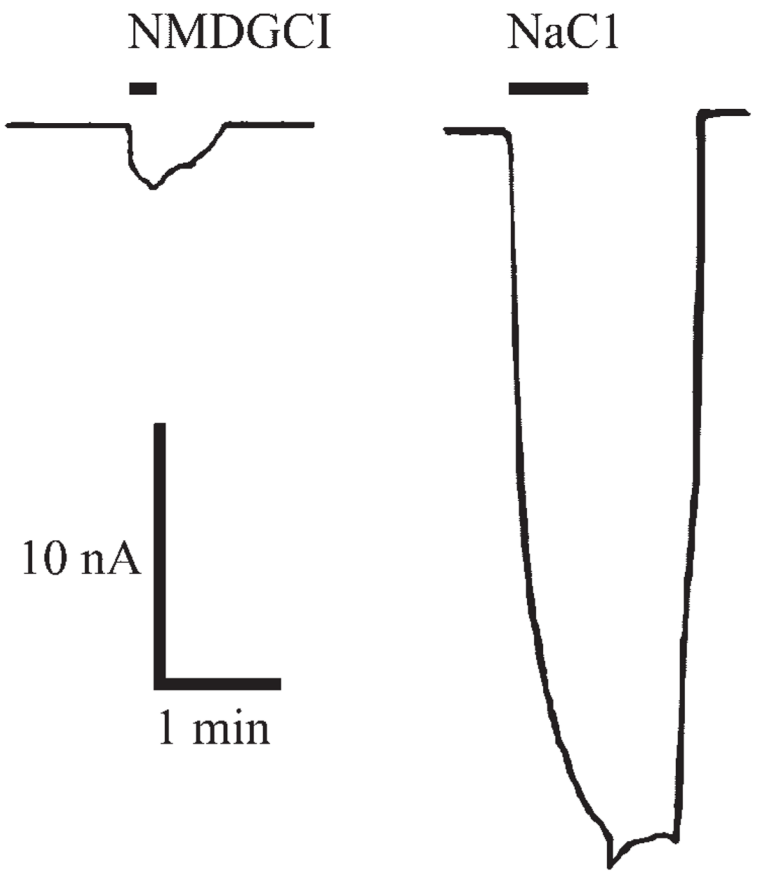

Fig 4.

Transport of butyryl-L-carnitine via hATB$^{0,+}$ in *Xenopus laevis* oocytes. Oocytes were injected with human ATB$^{0,+}$ cRNA, and, 6 days after injection, they were used in electrophysiological studies. Butyryl-L-carnitine (5 mM)-induced inward currents were monitored at −50 mV under voltage-clamp conditions in a *N*-methyl-D-glucamine (NMDG)-containing buffer (i.e., absence of Na^+) and an Na^+-containing buffer.

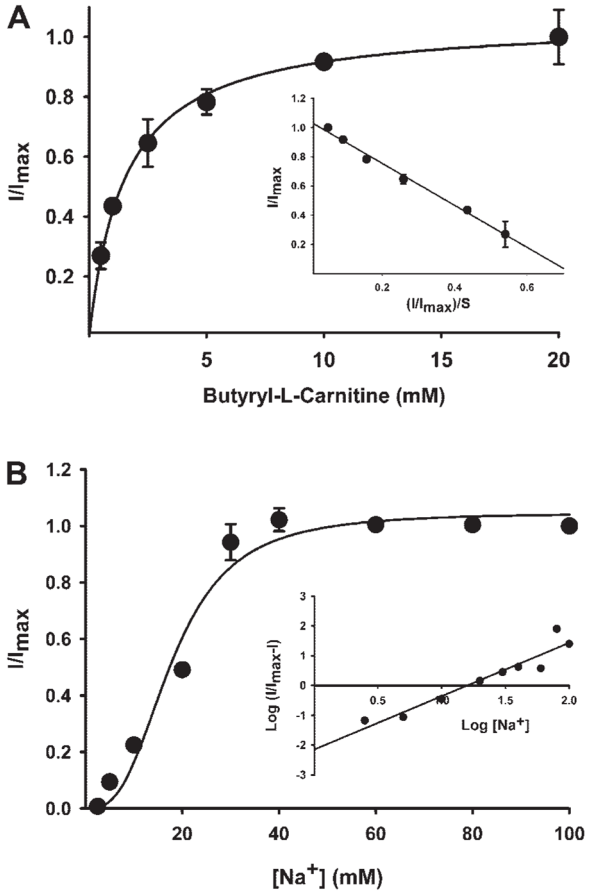

Fig 5.

A: characteristics of hATB$^{0,+}$-mediated butyryl-L-carnitine transport in *X. laevis* oocytes. Saturation kinetics for butyryl-L-carnitine-induced current in three different oocytes expressing human ATB$^{0,+}$. *Inset*: Eadie-Hofstee plot (I/I_{max}, butyryl-L-carnitine-induced current; S, butyryl-L-carnitine concentration in mM). *B*: Na^+ activation kinetics for butyryl-L-carnitine-induced current in three different oocytes expressing hATB$^{0,+}$. *Inset*: Hill plot. In *A* and *B*, the data were normalized by taking the maximal current induced in each oocyte as 1.

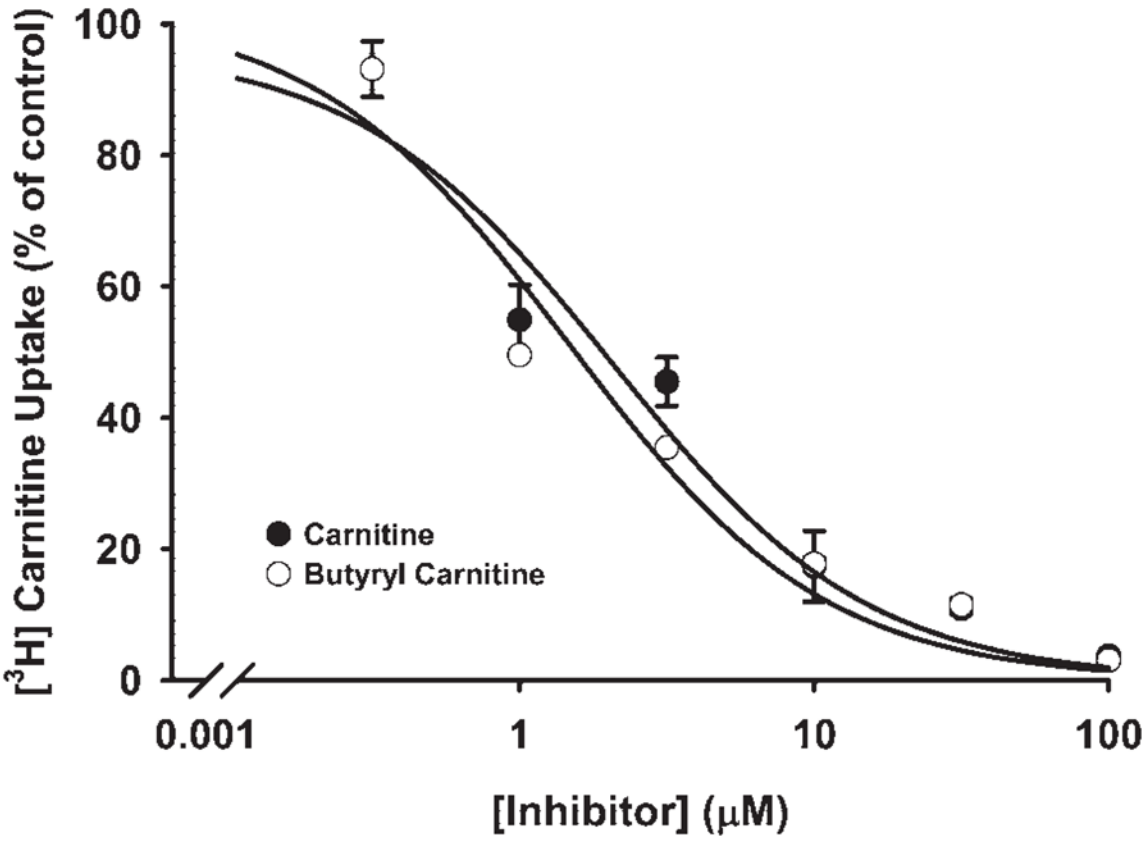

Fig 6.

Inhibition of hOCTN2-mediated [^{3}H]carnitine transport by L-carnitine and butyryl-L-carnitine in HRPE cells. HRPE cells were transfected with either vector alone or hOCTN2 cDNA. Transport activity was monitored by measuring the uptake of [^{3}H]carnitine (30 nM) with a 30-min incubation. The concentration of L-carnitine (●) and butyryl-L-carnitine (○) was varied over a range of 0–100 μM. Uptake in vector-transfected cells was subtracted from uptake in cDNA-transfected cells to calculate cDNA-specific uptake. Uptake in the absence of inhibitors was taken as 100%.

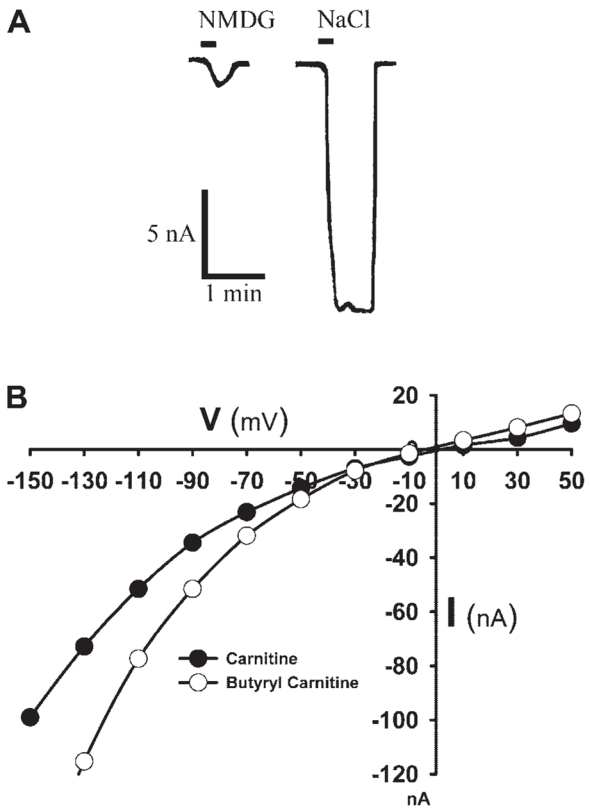

Fig 7.

Transport of butyryl-L-carnitine by hOCTN2 as assessed by induced currents in *X. laevis* oocytes expressing hOCTN2. *A*: induction of Na^+-dependent currents by butyryl-L-carnitine (10 μM) in oocytes under voltage-clamp conditions. The superfusion buffer contained either NMDG chloride (i.e., absence of Na^+) or NaCl. *B*: current (*I*)-voltage (*V*) relationship for L-carnitine and butyryl-L-carnitine (10 μM).

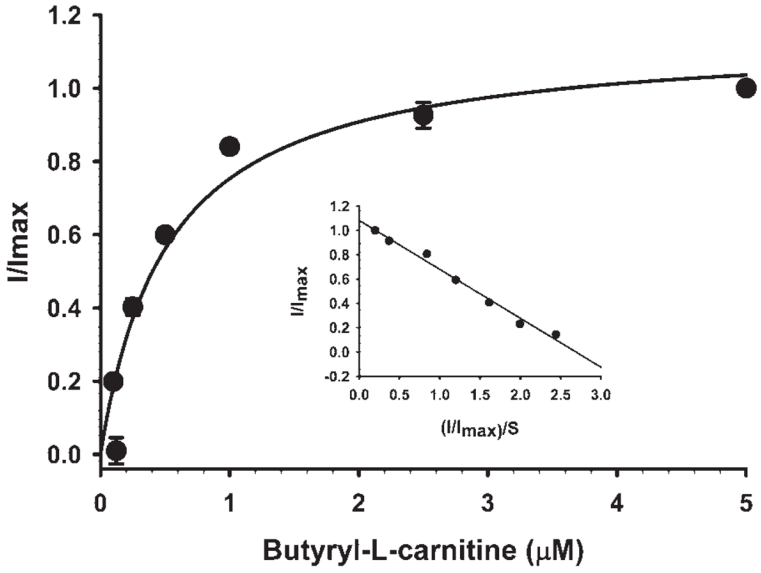

Fig 8.

Saturation kinetics for hOCTN2-mediated butyryl-L-carnitine transport in the *X. laevis* expression system. Saturation kinetics for butyryl-L-carnitine-induced current in three different oocytes expressing hOCTN2 was studied over a concentration range of 0.1-μM. *Inset*: Eadie-Hofstee plot; (I/I_{max}, butyryl-L-carnitine induced current; S, butyryl-L-carnitine concentration in μM). Data were normalized by taking the maximal current induced in each oocyte as 1.