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$G_{s\alpha}$-mediated regulation of the carnitine carrier in S49 lymphoma cells

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Abstract

Carnitine is essential for mitochondrial oxidation of long-chain fatty acids. Peripheral cells rely on plasma transport of carnitine which is taken up by an active mechanism in the plasma membrane. This project investigated the plasma membrane bound carnitine carrier in cultured S49 lymphoma cells. We investigated wild-type cells and two mutant cells lines showing deficient activity of adenylyl cyclase, cyc^- lacking and H21a containing a deficient $G_{s\alpha}$. Plasma membranes derived from cyc^- cells displayed six times more carnitine binding sites and a 1.35 times faster uptake rate than plasma membranes from wild-type cells. In vitro mixing of plasma membranes from cyc^- and wild-type cells transferred a factor reducing the number of expected carnitine binding sites by about 30%. Cyclic AMP could not substitute for wild-type membranes as the inhibitor of carnitine binding to plasma membranes derived from cyc^- cells. Cholera toxin induced ADP-ribosylation of $G_{s\alpha}$ causing activation of $G_{s\alpha}$ present in wild-type but not in cyc^- cells, further reducing carnitine uptake and carnitine binding to plasma membranes. Our findings thus supported the notion that $G_{s\alpha}$ by a mechanism not involving cyclic AMP inhibited cellular uptake of carnitine by reducing the number of available carnitine binding sites in plasma membranes.

Keywords: Carnitine cellular uptake; Carnitine plasma membrane binding; $G_{s\alpha}$; S49 lymphoma cell

1. Introduction

Carnitine is a low molecular weight compound essential for oxidation of long-chain fatty acids in the mitochondria [1]. Peripheral tissues depend on plasma transport of carnitine and maintain a 10–2000-fold carnitine concentration gradient over their plasma membrane [2–4]. An active carnitine uptake mechanism has been demonstrated in several tissues [2–10]. Reports suggest a negative influence on carrier-mediated carnitine uptake by LiCl presumably by interfering with the transmembrane sodium gradient [11], by cycloheximide inhibiting protein synthesis [12], by both SH-blocking or SH-binding reagents [11], and by diphtheria toxin [13]. Starvation [14] and prednisolone [15] seem to facilitate cellular uptake of carnitine. A cardiac carnitine binding protein has been identified in rat ventricular homogenates [16]. In a previous report [17] we have characterized binding of carnitine to plasma membranes derived from murine S49 lymphoma cells. Carnitine binding to plasma membranes was demonstrated to occur through a mechanism requiring ATP and magnesium ions and inhibited by genistein, a protein tyrosine kinase inhibitor [17].

We report here findings suggesting the $G_{s\alpha}$-protein to be a major negative regulator of carnitine cellular

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uptake and carnitine membrane binding in S49 lymphoma cells through a mechanism not involving cyclic AMP.

2. Materials and methods

2.1. Cell culture and membrane preparation

S49 lymphoma cells were cultivated in suspension culture and harvested as previously described [18]. Plasma membranes were purified by a discontinuous sucrose gradient and characterized using enzyme markers as reported [17]. The fraction between the 23% and 34% sucrose layer was denoted plasma membrane enriched fraction (PM_x) where the suffix x indicates the cell type wt, cyc$^-$ or H21a respectively fractionated.

2.2. [^{3}H]L-Carnitine binding to isolated PM

The radioligand binding assay was carried out [17] in 50 μl of a 20 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 120 mM NaCl supplemented with 1 mM $MgCl_2$, ^{3}H-labeled 0.4 μM–1 mM L-carnitine (4.4 million dpm), and various agents at indicated concentrations. Incubation was initiated by addition of 20 μg pre-warmed PM and stopped by snap freezing in liquid N_2. Non-specific carnitine binding was minimized by pelleting the membranes before the vacuum filtration through ultracentrifugation of the barely thawed incubation mixture. Tubes were thawed for 3 min, centrifuged $100\,000 \times g$ at 4°C for 1 h. The supernatant was discarded and the pellet resuspended in 750 μl binding assay buffer, filtered (BA 85, Schleicher and Schuell) and washed twice under reduced pressure at 4°C. The filters were dried and counted in 7.5 ml scintillation fluid (Ready Protein, Beckman). Non-specific binding of carnitine to membranes was defined by inclusion of 20 mM unlabeled carnitine in the incubation buffer. Equilibrium binding experiments were evaluated by non-linear regression analysis [19].

2.3. Cellular uptake of carnitine

Uptake in S49 lymphoma cells was performed in 500 μl of DMEM supplemented with 10% dialyzed horse serum, 0.4–200 μM L-carnitine and 74 kBq [^{3}H]L-carnitine and various agents at indicated concentrations as previously reported [9,17].

2.4. General analytical procedures

Succinate-cytochrome *c* reductase activity [20,21], carnitine palmitoyltransferase 1 [22,23], ouabain binding sites [24], β-adrenoceptors (specific [^{125}I]cyanopindolol binding), $G_{s\alpha}$ concentration, the $Al^{3+}/Mg^{2+}/F^-$-stimulated activity of the cyc$^-$ reconstituted adenylyl cyclase [25–28], and carnitine concentration [29,30] were determined as described. Treatment of cells and PM by cholera toxin were undertaken as published [31]. The actual ADP-ribosylation of $G_{s\alpha}$ induced by cholera toxin was checked

Table 1
Characterization of plasma membranes derived from wild-type, cyc$^-$, and H21a S49 lymphoma cells

	Wild-type	cyc$^-$	H21a
Ouabain binding sites (fmol/mg)	721 ± 64	641 ± 82	593 ± 112
β-Adrenoceptor binding (fmol/mg)	133 ± 21	168 ± 18	159 ± 24
$G_{s\alpha}$ concentration (pmol/mg)	38.6 ± 6.9	< 0.8 ± 0.6*	32.3 ± 8.1
Reconstituted adenylyl cyclase activity (pmol/mg/min)	321 ± 89	12 ± 8*	19 ± 11*
Carnitine palmitoyl transferase 1 (nmol/mg/min)	0.5 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
Succinate-cytochrome *c* reductase (nmol/mg/min)	0.6 ± 0.2	0.6 ± 0.1	0.5 ± 0.2

S49 lymphoma cells were subfractionated as described in Section 2 and the interphase fraction between 23% and 34% sucrose layers was used for assay for various marker enzyme activities. The membrane homogenate from wild-type cells applied on the gradient contained 43.1 ± 12.9 fmol/mg β-adrenoceptors, 13.7 ± 3.1 pmol/mg $G_{s\alpha}$, and 62.2 ± 9.2 nmol/mg/min succinate-cytochrome *c* reductase. The corresponding membrane homogenates from cyc$^-$ and H21a cells contained < 0.3 ± 0.4 and 12.1 ± 4.2 pmol/mg of $G_{s\alpha}$, respectively and 58.8 ± 7.9 and 64.1 ± 10.3 nmol/mg/min succinate-cytochrome *c* reductase activity, respectively.
*Statistically significant ($n = 8$; $P < 0.001$) differences in $G_{s\alpha}$ concentration (PM_{cyc-}) and in adenylyl cyclase (PM_{cyc-}, PM_{H21a}) reconstituted activity versus PM_{wt}.

using radiolabeled NAD^+ followed by gel electrophoresis and autoradiography as described [31]. Protein concentration was determined by the Bio-Rad microassay using bovine albumin as protein standard [32]. Results were expressed as mean ± S.D. (n = number of experiments).

3. Results

3.1. Binding of L-carnitine to PM

PM were prepared as reported [17] from S49 lymphoma cells. PM contained (Table 1) as judged by assay of the mitochondrial marker enzymes, carnitine palmitoyl transferase and succinate-cytochrome *c* reductase, negligible mitochondrial contamination while still retaining high concentrations of plasma membrane markers (ouabain binding sites, β-adrenoceptors). Two mutant S49 lymphoma cell lines having a defect adenylyl cyclase were utilized, the cyc^- mutant lacking the stimulatory G-protein, $G_{s\alpha}$, and the H21a strain having a deficient $G_{s\alpha}$ activity [33]. Both mutants were checked by assay of $G_{s\alpha}$ concentration and activity (Table 1).

Carnitine binding to PM was assessed using tritiated carnitine as reported [17]. PM_{wt} bound 475 pmol/mg membrane protein (Fig. 1) distributed on two binding sites displaying different affinities (Table 2). PM_{cyc-} (Fig. 1) and PM_{H21a} (data not shown) bound about sixfold more carnitine than PM_{wt}; all binding sites displayed a single affinity (Table 2). The difference in number of binding sites between PM from wild-type and mutant cell lines was decreased but nonetheless existed even when ATP was omitted from the incubation buffer (data not shown). These data suggested that the difference between PM from mutant and wild-type cells did not depend on the previously reported regulatory mechanism of carnitine binding operating through a protein tyrosine kinase.

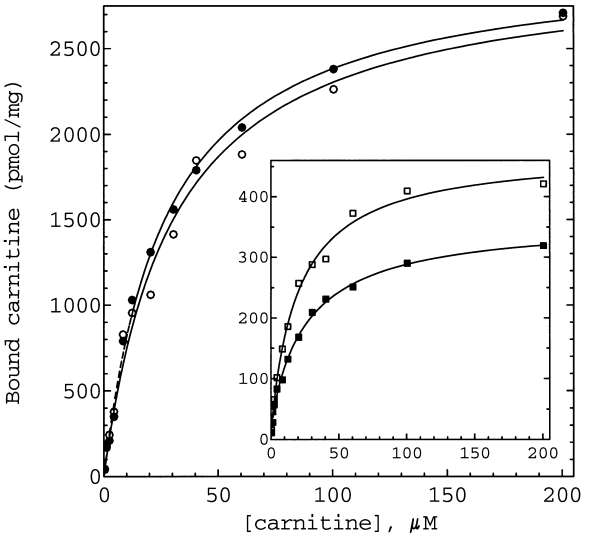

Fig. 1. Dose-response curves for carnitine binding to PM derived from wild-type and cyc^- cells. PM derived from wild-type (inset, □) and cyc^- (○) cells were incubated for 20 min at indicated concentrations of [^{3}H]L-carnitine in the absence (□, ○) and presence (■, ●) of dithiothreitol-activated cholera toxin and NAD^+ as described in Section 2. The number of carnitine binding sites and the corresponding dissociation constants were calculated [19] by non-linear regression analysis (Table 2). Each curve is based on a representative experiment run in triplicate.

The hypothesis that plasma membranes from wild-type cells contain a factor inhibiting carnitine membrane binding was investigated by experiments mixing PM_{cyc} and PM_{wt} (Table 3). Addition of PM_{wt} to

Table 2
Carnitine equilibrium binding to PM derived from wild-type, cyc^-, and H21a S49 lymphoma cells

	B_H (pmol/mg)	K_H (μM)	B_L (pmol/mg)	K_L (μM)
Wild-type cells	27 ± 8	1.2 ± 0.9	448* ± 51	21.3 ± 5.8
Wild-type cells+cholera toxin	47 ± 16	2.1 ± 1.2	313* ± 34	29.5 ± 6.8
cyc^- cells	–	–	2999 ± 132	28.9 ± 3.6
cyc^- cells+cholera toxin	–	–	3030 ± 103	27.2 ± 1.9
H21a cells	–	–	3012 ± 141	25.9 ± 3.1

PM derived from wild-type, cyc^-, and H21a cells were incubated for 20 min as described in Fig. 1 at different concentrations of [^{3}H]L-carnitine in the absence and presence of dithiothreitol-activated cholera toxin and NAD^+ as described in Section 2. The number of carnitine binding sites (B_H, H = high affinity sites, and B_L, L = low affinity sites; pmol/mg membrane protein) and the corresponding dissociation constants (K_H and K_L; μM) were calculated [19] by non-linear regression analysis.
*Values differ in a statistically significant way ($n = 6$, $P < 0.001$).

PM_{cyc-} prior to assaying carnitine binding to membranes reduced the number of available carnitine binding sites, not only below the expected sum of the respective membrane contributions, but to a level of about 30% less than in PM_{cyc-} alone. Addition of 100 μM cyclic AMP slightly attenuated carnitine binding in PM_{cyc-} but could not replace PM_{wt} as the inhibitory factor (Table 3).

3.2. Cellular carnitine uptake in S49 lymphoma cells

Carrier-mediated uptake of carnitine was saturable and followed Michaelis-Menten kinetics. γ-Butyrobetaine, a carnitine analogue, almost completely blocked the carrier-mediated carnitine uptake when added in tenfold excess and passive diffusion was estimated [34] at 50 μM carnitine to be 21 ± 4 amol/cell/h. Cellular uptake of carnitine was significantly higher ($P < 0.001$) in cyc^- cells than in wild-type S49 lymphoma cells (Fig. 2), carrier-mediated maximal carnitine uptake rate in wild-type S49 lymphoma cells was determined to be 173 ± 6 amol/cell/h ($n = 8$) whereas the maximal uptake rate in the cyc^- mutant S49 cell line was estimated to be 237 ± 7 amol/cell/h ($n = 4$). The K_m values were 21.9 ± 2.1 and 23.9 ± 1.9 μM for wild-type and cyc^- cells respectively, i.e. they did not differ significantly between the two cell types.

The carnitine concentration in media supplemented with 10% horse serum, i.e. under standard cell culture conditions, is approx. 2 μM. Determination of the intracellular concentration of carnitine in S49 lymphoma cells showed the cyc^- mutant cells to maintain a higher intracellular concentration of L-carnitine than wild-type cells. The intracellular concentration of carnitine was determined after 72 h at 2.1 μM carnitine in the utilized cell culture media to 70 ± 4 versus 53 ± 5 amol/cell for cyc^- and wild-type cells respectively ($n = 6$, $P < 0.01$). The volume of S49 lymphoma cells was determined to be 203 ± 31 fl ($n = 8$). The nuclei were estimated by area calculation in photographs to constitute about 62.3 ± 8.5% ($n = 8$) of total cellular volume leaving the cytoplasm

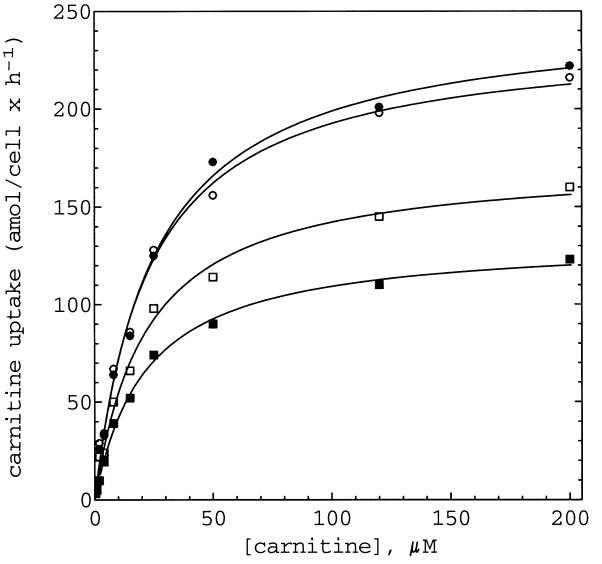

Fig. 2. Cellular carnitine uptake in untreated (□, ○) and cholera toxin pretreated wild-type and cyc^- S49 lymphoma cells. Wild-type (■) and cyc^- (●) cells were preincubated in cell culture media supplemented with 10 μg/ml cholera toxin for 1 h and then incubated at indicated concentrations of radiolabeled carnitine and analyzed for labeled carnitine as described in Section 2. Wild-type (□) and cyc^- (○) cells preincubated in cell culture media without cholera toxin were used as control. Non-saturable passive diffusion was subtracted from total cellular uptake of L-carnitine. Each curve is based on a representative experiment run in quadruplicate.

Table 3
Inhibition of carnitine binding to PM_{cyc-} by PM_{wt}

	No PM_{cyc-}	20 μg PM_{cyc-}	20+20 μg PM_{cyc-}
No PM_{wt}	132	102 178 ± 3112	174 098 ± 5809
20 μg PM_{wt}	10 631 ± 1231	78 911 ± 2652*	nd
20+20 μg PM_{wt}	20 447 ± 1983	nd	nd

Carnitine equilibrium binding experiments were performed utilizing membranes derived from wild-type (PM_{wt}), and cyc^- (PM_{cyc-}) S49 lymphoma cells. Carnitine binding assay was performed using respective membrane type or a mixture of both membrane types under conditions maximizing carnitine binding (200 μM carnitine, 20 min). No detergents were added. Results are expressed as counts/min. Predicted value for the mixture of 20 μg membranes of each type (marked with *) were 112 809 cpm; i.e. the actual binding obtained was statistically significantly ($P < 0.01$, $n = 5$) reduced by 30%. Addition of 100 μM cyclic AMP reduced binding by 3.6 ± 1.1%. nd, not done.

a volume of 73 ± 19 fl. The intracellular concentration of carnitine in the cytoplasm (we could not detect any carnitine in the nucleus) was thus calculated to be 959 ± 56 and 726 ± 70 μM in cyc$^-$ and wild-type cells respectively, i.e. the cyc$^-$ cells maintained an almost 500-fold concentration gradient over the plasma membrane. The rate of specific carrier-mediated uptake of carnitine at 2.1 μM carnitine in the media was determined to be 4.9 ± 0.8 and 8.9 ± 0.7 amol/cell/h ($n = 4$, $P < 0.001$) in wild-type and cyc$^-$ cells, respectively.

3.3. Effect of cholera toxin on carnitine membrane binding and cellular uptake

Cholera toxin specifically ADP-ribosylates the $G_{s\alpha}$-protein trapping the $G_{s\alpha}$ in its activated GTP-liganded state. PM were ADP-ribosylated by preincubation for 5 min prior to the carnitine equilibrium binding experiments in carnitine binding buffer supplemented with dithiothreitol-activated cholera toxin and NAD^+. Activated cholera toxin induced ADP-ribosylation of $G_{s\alpha}$ in PM from wild-type cells as verified using radiolabeled NAD^+ and subjecting the incubation mixture to SDS gel electrophoresis followed by autoradiography (data not shown). The presence of 50 μg/ml of activated cholera toxin attenuated the number of carnitine binding sites in PM_{wt} but left carnitine binding to PM_{cyc-} unperturbed (Fig. 2). The high affinity site for carnitine binding usually observable in PM_{wt} remained detectable in the presence of activated cholera toxin (Table 2). Unactivated cholera toxin did not influence carnitine binding.

Intact S49 lymphoma cells were preincubated for 1 h in ordinary cell culture media supplemented with 10 μg/ml of cholera toxin, resuspended in fresh media without cholera toxin, and cellular carrier-mediated carnitine uptake was measured at various carnitine concentrations. Only cells remaining viable after the cholera toxin treatment were utilized. Pretreatment by cholera toxin decreased statistically significantly the uptake rate of carnitine in wild-type ($P < 0.001$) but not in cyc$^-$ cells (Fig. 2); specific uptake rates were determined to be 133 ± 8 and 248 ± 7 amol/cell/h ($n = 4$), respectively. The K_m values were not significantly altered by the cholera toxin treatment.

4. Discussion

S49 lymphoma cells are of widespread use in signal transduction studies because of the availability of mutant cell lines displaying a defect adenylyl cyclase. Two of these mutant cell lines, the cyc$^-$ and H21a strains, were here found to display a quite substantial increase in both carnitine binding to PM and in cellular carnitine uptake rates as compared to wild-type cells. PM from the mutant cells lines displayed not only a sixfold increase in number of carnitine binding sites but also showed different binding characteristics with only one type of carnitine binding sites. Experiments utilizing mixing of PM from wild-type and cyc$^-$ cells demonstrated the presence of a transferable factor in PM_{wt} that induced a substantial inhibition of carnitine membrane binding in PM_{cyc-}. The reduction induced by adding PM_{wt} actually reduced the number of available carnitine binding sites to a level below the number of sites present in PM_{cyc-}, i.e. the difference between the two different membrane types could not solely be quantitative but must at least be qualitative. Cyclic AMP, based on the adenylyl cyclase defect in the mutant cells lines was an obvious factor to investigate. Direct addition of cyclic AMP to the PM induced a small reduction in the number of carnitine binding sites in PM but could not account for the difference between the two cell lines or the substantial inhibition observed when mixing the two membrane types. The absence of $G_{s\alpha}$-protein in membranes from cyc$^-$ cells may further be argued through its highly hydrophobic nature to cause some non-specific disruption of membranes changing carnitine membrane binding characteristics. This objection was refuted by the observation that the H21a mutant, having a deficient $G_{s\alpha}$-protein, showed carnitine membrane characteristics virtually identical to membranes from cyc$^-$ cells. The time frame, the absence of detergents, and the extent of the inhibitory effect made $G_{s\alpha}$, based on experience from of reconstitution of adenylyl cyclase by membrane extracts [28], a rather unlikely candidate for the role as a transferable inhibitor.

Although the cyc$^-$ mutant cell line lacks the $G_{s\alpha}$-protein [25,33], other hitherto unknown differences might be of importance for the transmembranous transport of carnitine. Cholera toxin induces ADP-ribosylation of $G_{s\alpha}$ enhancing the activity of G_s by

blocking the intrinsic GTPase activity in $G_{s\alpha}$ and thus leaving $G_{s\alpha}$ in its GTP-liganded state [33]. Preincubation of S49 lymphoma cells in cell culture media containing cholera toxin, attenuated the carnitine uptake rate in wild-type cells but left the uptake rate in cyc^- cells unperturbed, thus supporting the notion of a mechanistic connection between the $G_{s\alpha}$ activity and the cellular uptake mechanisms for carnitine. Membrane experiments utilizing cholera toxin showed that ADP-ribosylation of $G_{s\alpha}$ induced a decrease in the number of membrane binding sites for carnitine suggesting that increased $G_{s\alpha}$ activity induced inhibition of the carnitine carrier system. Cholera toxin did not affect carnitine binding to PM_{cyc-} or the cellular carnitine uptake rate in cyc^- cells indicating that the observed effects in wild-type cells were due to ADP-ribosylation of $G_{s\alpha}$ rather than non-specific toxic mechanisms. The observed differences between cyc^- and wild-type cells remained irrespective of the presence of ATP, indicating that the difference did not involve mechanisms related to the previously reported protein tyrosine kinase [17].

We thus conclude that $G_{s\alpha}$ seems to be a major negative regulator of the carnitine carrier by a mechanism not operating through adenylyl cyclase.

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