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Oleic acid inhibition of Na^+/D-glucose transport in isolated renal brush-border membranes: role of lipid physical parameters and *trans* Na^+-inhibition

Siegfried Wagner, Katharina Wenzel-Seifert, Lothar Volbracht, Detlef Sorgenfrei, Hans Ebel *

Institut für Klinische Physiologie, Klinikum Steglitz, Freie Universität Berlin, Hindenburgdamm 30, D-1000 Berlin 45, Germany

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Abstract

Inhibition of Na^+/D-glucose transport by oleic acid was investigated in renal brush-border membrane vesicles (BBMV). Lipid physical parameters were determined by spectrofluorometry. *cis*-Unsaturated C_{16}-C_{22} long-chain fatty acids (LCFA) as oleic acid reduced nonzero limiting anisotropy r_∞ with DPH and 12-AS as probes and decreased rotational correlation time ϕ of 12-AS. At 8 s and 15 s Na^+/D-glucose transport was competitively inhibited. A positive correlation existed between decrease in r_∞ (acyl chain order) or decrease in rotational correlation time ϕ (= increase in 'fluidity') and inhibition of Na^+/D-glucose transport. Except elaidic acid *trans* unsaturated and saturated LCFA had no effect on fluorescence anisotropy and Na^+/D-glucose transport. Per cent transport inhibition was unaffected by 0 voltage clamping and by FCCP. K_i for *trans* Na^+-inhibition of D-glucose transport was 29 mmol/l. Na^+-transport was stimulated by oleic acid, exceeding the K_i value for *trans* Na^+ inhibition. Conclusion: oleic acid inhibits Na^+/D-glucose transport by a decrease in lipid acyl chain order and an increase in 'fluidity', by *trans* Na^+-inhibition and presumably by a third *unknown* mechanism.

Key words: Oleic acid; Sodium ion/D-glucose transport; *trans* Sodium inhibition; Brush-border membrane; Membrane fluidity; Lipid–protein interaction; (Kidney)

1. Introduction

It is generally accepted that the activity of membrane bound proteins is dependent on the physical properties of the surrounding lipids. Amount and composition of the membrane phospholipids determine membrane lipid 'fluidity', membrane surface charge and membrane thickness. By these factors and by direct lipid–protein interactions membrane protein function might be affected. In case of the epithelial Na^+/D-glucose transporter several groups focussed on 'fluidity' as the main factor. They reported that in vitro an increase in membrane lipid 'fluidity' was associated with a decrease in Na^+/D-glucose transport of rat renal or intestinal brush-border membrane vesicles (BBMV) [1–5]. Vice versa, a decrease in BBMV lipid 'fluidity' caused in vitro by incorporation of cholesterol was correlated with an increased Na^+/D-glucose transport activity [6].

The inverse relationship between 'fluidity' and Na^+/D-glucose transport activity differs from that of most other membrane proteins. For instance, it is well known that the activity of enzymes as adenylate cyclase [7,8] and Na^+/K^+-ATPase [9,10] increases with increasing 'fluidity' in vitro. Furthermore, in BBMV the activity of transporter proteins as the Na^+-P_i cotransporter [11] and Ca^{2+} uptake [12] is directly correlated to 'fluidity'. Also, Na^+/H^+ antiport has been related to 'fluidity' [13], but others reported that it was unaffected by 'fluidity' [14].

The apparently adverse behaviour of the Na^+/D-glucose transporter has been explained by a faster

* Corresponding author. Fax: +49 30 7984141.

Abbreviations: BBMV, brush-border membrane vesicles; DPH, 1,6-diphenyl-1,3,5-hexatriene; FCCP, carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone; LCFA, long-chain fatty acids; 2-AS, 7-AS, 12-AS, *n*-(9-anthroyloxy)-stearic acid; 16-AP, 16-(9-anthroyloxy)palmitic acid.

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collapse of the Na^+ gradient [2,4,15], which is the driving force for the Na^+/D-glucose transport. The 'Na^+ gradient collapse theory' has been contradicted by Friedlander et al. [16,17]. The authors showed that 'fluidization' of cultured renal epithelial cells by benzyl alcohol inhibited the Na^+-dependent transport of a hexose. In addition, it has been demonstrated that in intestinal BBMV also Na^+-dependent proline transport decreases with increasing 'fluidity' [18].

The aim of our study was to analyse the inhibitory effect of oleic acid on Na^+/D-glucose transport in renal BBMV. It will be shown 1) that the oleic acid induced inhibition of Na^+/D-glucose transport is strongly correlated to lipid order parameter as well as to lipid rotational mobility ('fluidity'), 2) that Na^+-uptake is increased and 3) that the increased intravesicular Na^+ exceeds the K_i for *trans* Na^+ inhibition.

2. Materials and methods

Materials

D-[6(n)-^{3}H]Glucose (0.93–1.85 TBq/mmol), [1-^{14}C]oleic acid (1.48–2.22 GBq/mmol) and (U-^{14}C)-labeled palmitic acid (> 18.5 GBq/mmol) were obtained from NEN Research Products/Du Pont de Nemour (Dreieich, Germany), ^{22}NaCl (3.7–37 GBq/mg Na) was from Amersham-Buchler (Braunschweig, Germany). DPH and *n*-(9-anthroyloxy) fatty acids were purchased from Molecular Probes (Seattle, WA, USA) and Filter Count^R was from Canberra Packard GmbH (Frankfurt, Germany). Oleic acid methyl ester was from FLUKA (Neu-Ulm, Germany). All other chemicals were obtained from Merck (Darmstadt, Germany) or from Sigma (Taufkirchen, Germany). Nitro cellulose filters (HAWP 02500) were from Millipore GmbH (Eschborn, Germany).

Isolation of renal brush-border membranes

Male Sprague-Dawley rats weighting 180–220 g were killed by decapitation and their kidneys were rapidly placed on ice. BBMV were prepared from thin renal cortex slices using a Mg^{2+} precipitation method [19,20]. Final BBMV were suspended at a concentration of 10 mg protein/ml in isotonic mannitol-Hepes-Tris (MHT_i) buffer (300 mmol/l mannitol, 10 mmol/l Hepes-Tris (pH 7.4)) for transport studies and in hypotonic MHT_h buffer (100 mmol/l mannitol) for fluorometry to reduce light scattering. The quality of the preparation was routinely determined by assay of marker enzymes. Alkaline phosphatase and aminopeptidase as markers for BBMV were enriched 12–15 fold. The corresponding values for Na^+/K^+-ATPase (basolateral membranes), succinate dehydrogenase (mitochondria), β-glucuronidase (lysosomes) and glucose-6-phosphatase (endoplasmic reticulum) ranged from 0.2 to 1.4. The isolated BBMV were stored in liquid nitrogen up to one week until use.

Enzyme and protein determinations

Aminopeptidase (EC 3.4.11), alkaline phosphatase (EC 3.1.3.1), Na^+/K^+-ATPase (EC 3.6.1.3), succinate dehydrogenase (EC 1.3.99.1), glucose-6-phosphatase (EC 3.1.3.9) were assayed by spectrophotometric methods as described earlier [21]. β-Glucuronidase (EC 3.2.1.31) was determined according to Stahl and Touster [22]. Protein was determined according to the method of Lowry et al. using bovine serum albumin as standard [21].

Fatty acid incorporation

Unsaturated LCFA were readily dissolved in 80% (v/v) ethanol. Saturated LCFA were dissolved in pure ethanol, warmed up to 50°C and sonicated with 30 Watts for 6 min, resulting in a clear microdispersed suspension. BBMV were incubated for 30 min at 37°C with freshly prepared fatty acid (500 nmol fatty acid/mg protein). Control BBMV were incubated with ethanol (0.5% v/v or less). To remove unbound fatty acids BBMV were sedimented at $48000 \times g$ and resuspended in MHT buffer for fluorescence or transport studies. For measurement of fatty acid incorporation into BBMV, membranes were incubated with ^{14}C-labeled oleic acid or palmitic acid (500 nmol/mg protein; 3.7 kBq).

Fluorescence spectroscopy

Fluorescence spectroscopy with DPH and *n*-(9-anthroyloxy) fatty acid derivates as probes was used to determine membrane lipid physical parameters. r_∞, the limiting hindered fluorescence anisotropy was used as a measure of lipid acyl chain orientational order. r_∞ is related to the orientational order parameter S by $S^2 = r_\infty/r_o$ with r_o as the maximal fluorescence anisotropy [24,25]. In membranes where the rotations of DPH are largely hindered and anistropic the fluorescence anisotropy r_s is mainly determined by r_∞ [24–27] so that r_s of DPH can simply be used as an indicator for lipid acyl chain order.

Unlike DPH, anthroyloxy fatty acids probes such as 12-AS with low r_∞ values are less influenced by membrane order so that r_s mainly reflects the rotational movement of the probe [28]. In case of 12-AS we used the rotational correlation time ϕ as a measure of lipid acyl chain rotational mobility ('fluidity').

Steady-state polarized fluorescence anisotropy r_s, fluorescence lifetimes and differential polarized fluorescence lifetimes were measured on an SLM 4800 spectrofluometer (SLM Instruments, Urbana, IL) interfaced with a personal computer. Fluorescence intensity was measured with the T format and anisotropy was calculated using equation $r_s = ((I_v/I_h)_v - (I_v/$

$I_h)_h/((I_v/I_h)_v + 2(I_v/I_h)_h)$, were $(I_v/I_h)_v$ and $(I_v/I_h)_h$ are the ratios of vertically to horizontally polarized emission light excited with vertically and horizontally polarized light, respectively. Fluorescence lifetimes were determined by a phase and intensity modulation technique as introduced by Spencer and Weber [29,30]. Since, at least in case of oleic acid, there was no change in the overall lifetime of the excited state, no attempts have been performed for lifetime heterogeneity analysis. Nonzero limiting anisotropy r_∞ and rotational correlation time ϕ were determined according to Lakowicz et al. [26].

Stock solutions of 2 mmol/l DPH in tetrahydrofurane and of 1 mmol/l anthroyloxy fatty acids in 80% v/v ethanol were stored. Before experiment the probes were diluted into 100 mmol/l MHT_h buffer and stirred for 60 min or 5 min, respectively, to remove solvent. BBMV suspensions in MHT_h buffer (100 μg/ml protein) were incubated for 30 min at 37°C with 1 μmol/l DPH or for 15 min at 25°C with 2 μmol/l *n*-AS. Subsequently BBMV were sedimented at $48000 \times g$ and resuspended in 100 mmol/l MHT_h buffer. As maximal limiting anisotropy r_o values of 0.39 for DPH [26,31] and 0.285 for 12-AS [28] were used. Excitation wavelengths were set to 360 nm for DPH and to 365 nm for *n*-AS. Light scattering was minimized to less than 0.5% of the emission by using a 4 nm band pass in the excitation monochrometer, a Schott KV 418 sharp cuton filter and by a low protein concentration in the cuvette (100 μg/ml). All samples were thermoequilibrated at 25°C.

Transport measurements

Uptake of D-[^{3}H]glucose by BBMV was measured at 25°C with a rapid membrane filtration technique as originally described by Hopfer et al. [32]. BBMV suspended in isotonic MTH_i buffer were preincubated at 25°C for 5 min. The reaction was initiated by adding BBMV (100 μg protein per assay) to reaction mixture to give 100 mmol/l manitol, 10 mmol/l Hepes-Tris (pH 7.4), 100 mmol/l NaCl or KCl, 0.1 mmol/l D-glucose (49 kBq D-[^{3}H]glucose) in a volume of 20 μl. Uptake was terminated first by dilution with 1 ml ice-cold stop solution (150 mmol/l NaCl, 10 mmol/l Tris-HCl (pH 7.4) and 0.2 mmol/l phlorizin) and then by rapid filtration on prewetted 0.45 μm nitrocellulose filters. After washing with 9 ml ice-cold stop solution the wet filters were dissolved in Filter-Count^R and analysed for label incorporation in a liquid scintillation counter.

In one series of experiments chemical voltage clamping was performed by equal internal and external 100 mmol/l KSCN plus valinomycin (12.5 μg/mg protein) as described by Turner and Moran [33].

In another series of experiments, 10 μmol/l of the ionophore FCCP was included in the assay medium to eliminate a H^+ gradient created by a possible protonophorous action of oleic acid.

For measurement of D-glucose uptake under equilibrium exchange conditions the BBMV were preequilibrated with a solution identical to the reaction medium. The experiment was started by addition of radiolabeled D-glucose.

For Na^+ uptake studies two different conditions were employed. First under an $i > o$ H^+ gradient BBMV were preequilibrated in a solution containing 150 mmol/l KCl and 20 mmol/l Mes-KOH (pH 5.5). Then, the BBMV solution was added to incubation medium to give 150 mmol/l KCl, 15 mmol/l Hepes-KOH (pH 7.5) and 5 mmol/l NaCl (74 kBq ^{22}Na). Uptake was terminated by addition of 1 ml ice-cold stop solution (150 mmol/l KCl, 15 mmol/l Hepes-KOH (pH 7.5) and 0.1 mmol/l amiloride) followed by rapid filtration on 0.45 μm filters.

In a second series Na^+ uptake was measured under glucose transport conditions. BBMV soluted in isotonic MTH buffer were added to an incubation medium containing 200 mmol/l mannitol, 20 mmol/l Hepes-Tris (pH 7.4) and 50 mmol/l NaCl (148 kBq ^{22}Na) with or without 0.1 mmol/l glucose. The stop solution was the same as above. All uptake data were corrected for unspecific tracer binding to the filters. Experiments were done in triplicate in at least four indepedent experiments.

Statistical analysis

Data were expressed as means ± S.E. and statistical differences were determined by Student's two-tailed paired *t*-test. $P < 0.05$ was considered significant. Regression lines were calculated by the method of the least squares.

3. Results

Fatty acid incorporation

Fatty acid incorporation was measured using two types of fatty acids: oleic acid as an example of unsaturated LCFA and palmitic acid as a saturated LCFA. When BBMV were incubated with 500 nmol/mg protein of ^{14}C-labeled fatty acid the incorporation rate was 2.28 ± 0.43 nmol/mg protein for oleic acid and 2.01 ± 0.51 nmol/mg protein for palmitic acid (mean ± S.E., $n = 14$). In renal BBMV the content of oleic acid or palmitic acid has been reported to be in the range of about 50 pmol/mg protein or 150 pmol/mg protein, respectively [34]. Thus, the fatty acid content of our BBMV must have been dramatically increased. Furthermore, the unsaturated and the saturated LCFA were incorporated to the same extent. Hence, the different effects of both types of fatty acids on fluores-

cence parameters and transport cannot be explained by differing amounts of binding to BBMV.

Decrease of fluorescence anisotropy by oleic acid

In a series of experiments r_s was 0.2598 ± 0.0039 (n = 10) for DPH and 0.1241 ± 0.0008 (n = 8) for 12-AS as probe, which is in the range previously reported by others [35,36]. Oleic acid at a concentration of 500 nmol/mg protein significantly reduced r_s to 0.1926 ± 0.0041 with DPH (n = 10) and to 0.0975 ± 0.0026 with 12-AS (n = 10), which is a reduction by 26% or 21%, respectively. For baby hamster kidney cells it has been reported that oleic acid reduced r_s of DPH by 19% [37]. After centrifugation and resuspension of our oleic acid treated BBMV the change of r_s was consistent for more than 24 h, pointing to a stable and irreversible incorporation of the fatty acid.

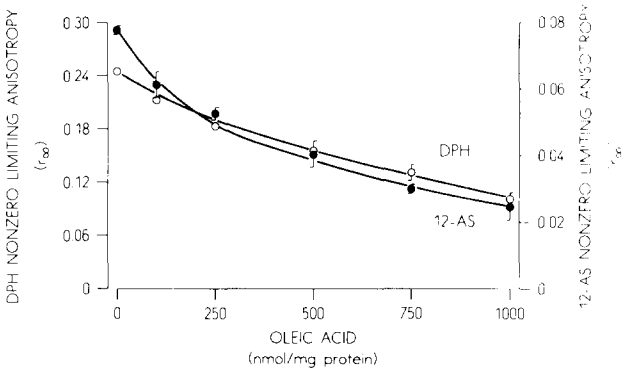

Fig. 1. Effect of varying doses of oleic acid on nonzero limiting anisotropy r_∞ with DPH (○) and 12-AS (●) as probes. BBMV were treated at 37°C for 30 min with oleic acid (500 nmol/mg protein). In all experiments unbound oleic acid was removed by resuspension of BBMV in MHT buffer (100 μg protein/ml). r_∞ was determined at 25°C according to Lakowicz et al. [31]. Mean ± S.E., n = 9.

Concentration dependency of the effect of oleic acid on nonzero limiting fluorescence anisotropy and rotational correlation time

Fig. 1 shows that increasing amounts of oleic acid progressively reduced r_∞ with both DPH and 12-AS as probes. At 500 nmol oleic acid/mg protein r_∞ decreased by 32% with DPH and by 42% with 12-AS. The maximally effective concentration of oleic acid was in the range of 2 μmol/mg protein. The increase in r_∞ of both probes indicates a decrease in the degree of order of the reported segments of the lipid acyl chains.

Fig. 2 shows that with 12-AS as probe ϕ was decreased concentration dependently decreased by oleic acid. At 500 nmol oleic acid/mg protein ϕ was significantly decreased by 33% from 3.06 ± 0.04 ns to 2.05 ± 0.05 ns. This indicates an increase in lipid acyl chain rotational mobility ('fluidity').

Table 1
Effect of acyl chain variations of LCFA on r_s and phase liftetime (τ_p) of DPH using exiting light modulated at a frequency of 18 MHz. Mean ± S.E. n = number of experiments, * = $P < 0.05$

Group		n	Lifetime τ_p (ns)	Anisotropy	
				r_s	change r_s (per cent)
Control		34	9.67 ± 0.11	0.2640 ± 0.0010	
Saturated fatty acids					
16:0	palmitic	14	10.12 ± 0.16 *	0.2595 ± 0.0032	−2
18:0	stearic	13	10.07 ± 0.23	0.2561 ± 0.0068	−3
19:0	nonadecyclic	8	10.23 ± 0.27	0.2562 ± 0.0022	−3
20:0	arachidic	7	10.28 ± 0.28	0.2633 ± 0.0034	+1
Unsaturated fatty acids					
16:1, *cis*-9	palmitoleic	8	8.93 ± 0.19 *	0.1799 ± 0.0097	−33
16:1, *trans*-9	palmitoelaidic	10	9.76 ± 0.05	0.2263 ± 0.01	−14
18:1, *cis*-6	petroselinic	18	9.65 ± 0.28	0.1708 ± 0.040 *	−35
cis-9	oleic	34	9.60 ± 0.13	0.1818 ± 0.0043 *	−31
	oleic, methyl ester	9	8.18 ± 0.14 *	0.1551 ± 0.0079 *	−41
trans-9	elaidic	11	9.43 ± 0.11 *	0.2552 ± 0.0063 *	−3
18:2, *cis*-9,12	linoleic	15	8.87 ± 0.39 *	0.1480 ± 0.0045 *	−44
trans-9,12	linolelaidic	8	8.88 ± 0.15 *	0.1913 ± 0.0043 *	−28
18:3, *cis*-9,12,15	linolenic	8	8.38 ± 0.14 *	0.1941 ± 0.0045 *	−27
20:1, *cis*-11	*cis*-11-eicosenoic	8	9.80 ± 0.08	0.1646 ± 0.0031 *	−38
20:2, *cis*-11,14	*cis*-11,14-eicosadienoic	8	8.28 ± 0.12 *	0.1910 ± 0.0011 *	−28
20:3, *cis*-8,11,14	*cis*-8,11,14-eicosatrienoic	8	8.43 ± 0.17 *	0.1496 ± 0.0081 *	−43
20:4, *cis*-5,8,11,14	*cis*-5,8,11,14-eicosatetraenoic	8	7.44 ± 0.15 *	0.1558 ± 0.0642 *	−41
20:5, *cis*-5,8,11,14,17	*cis*-5,8,11,14,17-eicosatetraenoic	8	7.96 ± 0.18 *	0.1910 ± 0.0034 *	−28
22:1, *cis*-13	erucic	8	10.27 ± 0.17 *	0.2480 ± 0.0028 *	−6
22:4, *cis*-15	nervonic	8	9.74 ± 0.16	0.2577 ± 0.0027 *	−2

Effects of acyl chain variation on fluorescence anisotropy and lifetimes

The effects of acyl chain variations on r_s and lifetimes were systematically investigated with DPH as probe (Table 1). The decrease of r_s by unsaturated LCFA (500 nmol/mg protein) was related to the *cis*- or *trans*-configuration of the double bound. In the *cis*-configuration r_s was reduced between 16:1 and 20:1. Longer acyl chains than 22:1 did not change r_s. In C_{18} fatty acids the acyl chain position of the double bound had only minor effects on r_s. In C_{20} unsaturated fatty acids no clear relationship between decrease in fluorescence anisotropy and number of double bounds could be detected. Methylation of the acyl chain, as shown with oleic acid, enhanced the decrease of r_s. *trans*-Unsaturated LCFA as palmitielaidic acid and elaidic acid showed only a minor or no effect on r_s. Saturated fatty acids as palmitic acid, stearic acid, nonadecanoic acid and arachidic acid were without effect on r_s.

It should be noted that some of the fatty acids slightly reduced the lifetimes compared to controls. Hence, a change in the dielectric constant could contribute, at least in part, to a change of r_s. However, a decrease of r_s was also seen when there was no decrease in fluorescence lifetime, e.g., in case of petroselinic acid and of oleic acid.

Monitoring fluorescence anisotropy from different membrane regions

To monitor the oleic acid induced decrease of r_s in different transversal membrane regions a set of *n*-(9-anthroyloxy) fatty acid derivatives was employed. At least when embedded in phospholipid bilayers these probes report the microenvironments at a graded series of depths from the surface to the middle and inner region of the membrane leaflet [38,39]. Oleic acid treated BBMV (500 nmol/mg protein) were labeled with 2-, 7-, 12-AS and 16-AP as probes. With increasing carbon number of the fatty acid probes r_s was progressively decreased by oleic acid (Fig. 3). Maximal disordering effects of oleic acid were observed between C_{12} and C_{16} which are located in the middle region of the membrane leaflet.

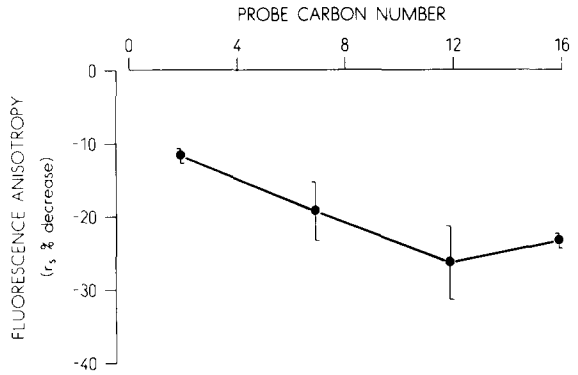

Fig. 3. Decrease of r_s (per cent) in BBMV treated with 500 nmol oleic acid/mg protein, monitored with a set of *n*-(9-anthroyloxy) fatty acid derivates with different carbon numbers. Mean ± S.E., $n = 8$.

Inhibition of D-glucose transport by oleic acid

The effect of oleic acid on Na^+-dependent D-glucose transport was usually investigated in MHT_i buffer. Fig. 4 illustrates the time course of D-glucose transport in control and oleic acid-treated BBMV (500 nmol/mg protein) under initial Na^+ o > i gradient conditions. Both vesicle preparations exhibited typical overshoot curves. In controls the peak uptake rate of D-glucose averaged 722 ± 44 pmol/mg protein per 15 s ($n = 22$). Pretreatment with oleic acid reduced D-glucose accumulation by 29% after 8 s and by 38% after 15 s. The reduction of Na^+/D-glucose transport by 7% after 4 s was not significant. Equilibrium D-glucose uptake was unaffected by oleic acid, indicating identical intravesicular volumes of control and oleic acid pretreated

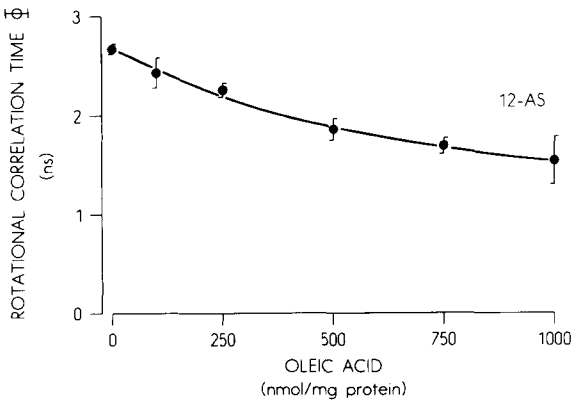

Fig. 2. Effect of varying doses of oleic acid on the rotational correlation time ϕ of 12-AS in BBMV. ϕ was determined according to Lakowicz et al. [31]. Mean ± S.E., $n = 9$.

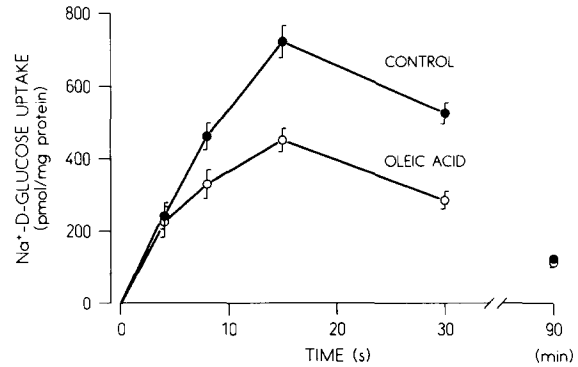

Fig. 4. Time-course of Na^+/D-glucose transport at 25°C in control and oleic acid treated (500 nmol/mg protein) BBMV. The initial o > i gradients were Na^+ = 100 mmol/l and D-glucose = 0.1 mmol/l. Mean ± S.E., $n = 11$.

Table 2
Effect of tonicity on oleic acid inhibition of Na^+/D-glucose transport. Transport was measured in isotonic MHT_i or in hypotonic MHT_h buffer. Mean ± S.E. n = number of experiments, * = $P < 0.05$

Group	n	Na^+/D-Glucose transport (nmol/mg protein per 15 s)		
		Control	Oleic acid	Change (per cent)
Isotonic	9	486 ± 61	246 ± 49 *	−39 ± 7
Hypotonic	9	651 ± 96	410 ± 79 *	−37 ± 8

BBMV (1.41 ± 0.09 μl/mg protein vs 1.32 ± 0.12 μl/mg protein). Na^+-independent D-glucose uptake, as determined in the presence of an inwardly directed K^+ gradient, was similar in both control and oleic acid treated BBMV (data not shown).

In one series of experiments the inhibition of Na^+/D-glucose transport by oleic acid was measured in MHT_h buffer as used in our fluorescence studies. Table 2 shows that the per cent inhibition of Na^+/D-glucose transport was the same in MHT buffers of different tonicity. Thus, comparison of the effects of LCFA on transport in isotonic media to fluorescence results in hypotonic media is justified.

Effect of acyl chain variation on D-glucose transport inhibition

The effect of different types of LCFA (500 nmol/mg protein) on Na^+-dependent D-glucose transport (Table 3) was compared with the effect on lipid order parameter and rotational mobility. Saturated C_{16} to C_{18} fatty acids which did not modulate lipid order did not significantly effect Na^+/D-glucose transport. Unsaturated *cis* C_{18} to C_{20} fatty acids inhibited Na^+/D-glucose transport by about 30–40%. As shown with oleic acid, methylation did not abolish the inhibition of Na^+/D-glucose transport. Unsaturated *trans*-configured LCFA which did not modulate lipid order and rotational mobility also did not inhibit Na^+/D-glucose transport. Interestingly, in case of elaidic acid change in r_s did not correlate with Na^+/D-glucose transport rate. Elaidic acid that did not effect r_s stimulated Na^+/D-glucose transport activity.

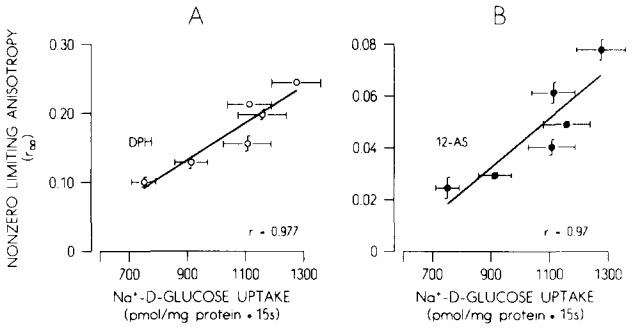

Fig. 5. Correlation between r_∞ with DPH (A) and 12-AS (B) as probes and Na^+/D-glucose transport. BBMV were treated with varying doses of oleic acid. Mean ± S.E., $n = 9$. r = correlation coefficient.

Relationship between lipid order or rotational mobility and D-glucose transport

To establish a relationship between lipid acyl chain order (r_∞) or lipid acyl chain rotational mobility (ϕ) and Na^+/D-glucose transport, transport activity was measured at different concentrations of oleic acid. With DPH and 12-AS as probes a strong correlation of r_∞ and Na^+/D-glucose transport activity was found (Fig. 5). With decreasing lipid order the Na^+/D-glucose transport rate decreased. With 12-AS as probe a strong correlation of ϕ with Na^+/D-glucose transport was demonstrated (Fig. 6).

Table 3
Effect of acyl variations of LCFA on Na^+/D-glucose transport in BBMV. Mean ± S.E. n = number of experiments, * = $P < 0.05$

Group		n	Na^+/D-Glucose transport (nmol/mg protein per 15 s)	change (per cent)
Control		22	722 ± 44	
Saturated fatty acids				
16:0	palmitic	9	636 ± 53	−12
18:0	stearic	9	634 ± 69	−12
Unsaturated fatty acids				
16:1, *trans*-9	palmitelaidic	8	716 ± 48	< −1
18:1, *cis*-9	oleic	22	451 ± 32 *	−38
	oleic, methyl ester	9	405 ± 79 *	−44
trans-9	elaidic	12	895 ± 65 *	+24
18:2, *cis*-9,12	linoleic	8	484 ± 50 *	−33
trans-9,12	linolelaidic	15	693 ± 47	−4
20:2, *cis*-11,14	*cis*-11,14-eicosadienoic	7	511 ± 134 *	−29
20:4, *cis*-5,8,11,14	*cis*-5,8,11,14-eicosatetraenoic	7	479 ± 89 *	−34
20:5, *cis*-5,8,11,14,17	*cis*-5,8,11,14,17-eicosatetraenoic	7	436 ± 86 *	−40

Competitive inhibition of D-glucose transport

The kinetic of the effect of oleic acid on Na^+/D-glucose transport was analysed. Since at 4 s the effect of oleic acid was not significant, D-glucose uptake rate was measured at 8 s with different glucose concentrations in control and oleic acid treated BBMV (500 nmol/mg protein).

Previous investigators provided kinetic and molecular evidence for the existence of two renal Na^+/D-glucose cotransporters in the mammalian kidney: a low affinity transporter in the outer cortex and a high affinity transporter in the outer medulla [38,40]. Therefore, we only obtained a linear Lineweaver-Burk plot in the range of 50 μmol/l to 1000 μmol/l of D-glucose when BBMV were prepared from very thin slices of the outer cortex (Fig. 7). Pretreatment of BBMV with 500 nmol oleic acid/mg protein increased the apparent K_m value nearly 1.5 fold (from 0.29 mmol/l to 0.43 mmol/l) while the apparent V_{max} values were not significantly different, which is a 'competitive inhibition'.

Increase in Na^+ transport by oleic acid

The effect of oleic acid on Na^+ transport was investigated. At first Na^+-transport was measured under the condition of an i > o proton gradient (i = pH 5.8, o = pH 7.5), which is optimal for the Na^+/H^+ exchanger [41]. At an o > i Na gradient of 5 mmol/l, 500 nmol oleic acid/mg protein significantly stimulated Na^+ transport by about 55% (Fig. 8A).

Since the effect of oleic acid on Na^+/D-glucose transport was measured at pH equilibrium and in presence of D-glucose, the effect of oleic acid on Na^+ transport was examined also under this condition ($pH_i = pH_o$ at pH 7.4, D-glucose o > i = 100 μmol/l, Na^+ o > i = 50 mmmol/l). Fig. 8B shows in accord with previous reports that D-glucose stimulated Na^+-transport [42,43]. Oleic acid (500 nmol/mg protein) significantly increased both the D-glucose-independent Na^+/H^+ exchange transport system and the D-glucose-dependent Na^+ transport. In oleic acid treated BBMV Na^+ transport amounted about 130 nmol/mg protein per 10 s.

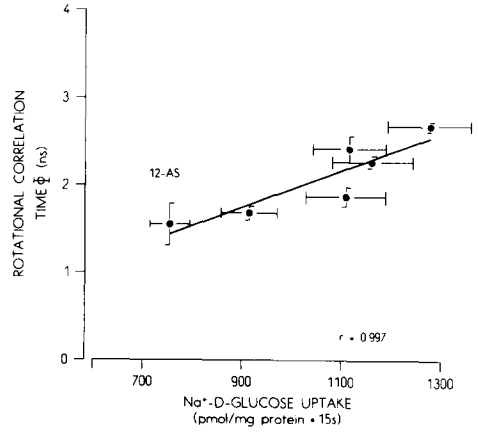

Fig. 6. Correlation between ϕ of 12-AS and Na^+/D-glucose transport. BBMV were treated with varying doses of oleic acid. Mean ± S.E., $n = 9$, r = correlation coefficient.

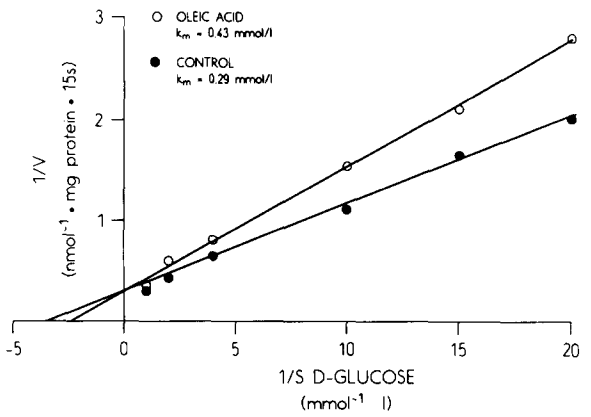

Fig. 7. Lineweaver-Burk plot of Na^+/D-glucose transport in control and oleic acid treated BBMV (500 nmol/mg protein) from outer kidney cortex. Mean ± S.E., $n = 4$.

trans Na^+ inhibition of D-glucose transport

The effect of Na^+ on D-glucose transport was investigated. D-Glucose transport was measured under Na^+ equilibrium. In Na^+-equilibrated BBMV the effect of oleic acid (500 nmol/mg protein) on D-glucose transport disappeared. The transport rate at 15 s, which is the peak value under glucose plus Na^+ gradient conditions, was 146 ± 11 pmol/mg protein for control and 140 ± 11 pmol/mg protein for oleic acid treated BBMV ($n = 15$).

The effect of *trans* Na^+ inhibition on Na^+/D-glucose transport was quantitated. *trans* Na^+ concentrations of 50 μmol/l and 100 μmol/l D-glucose resulted in decreased activities of D-glucose transport (Fig. 9).

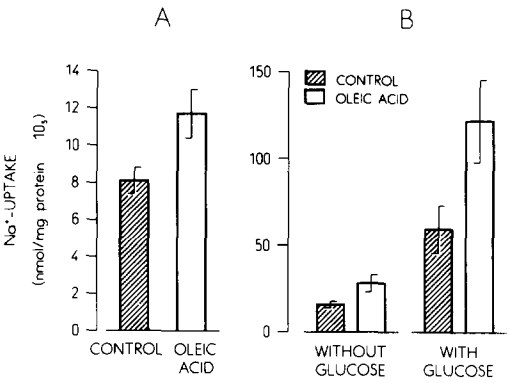

Fig. 8. Effect of oleic acid (500 nmol/mg protein) on Na^+-transport of BBMV (100 μg protein per assay) (A) under a H^+ gradient with $pH_i > pH_o$ at $pH_i = 5.8$ and $pH_o = 7.5$ and an initial Na^+-gradient of 5 mmol/l and (B) under H^+ equilibrium of pH = 7.4 and D-glucose transport conditions with initial o > i gradients of $Na^+ = 50$ mmol/l and D-glucose 0,1 mmol/l. Mean ± S.E. $n = 12$ in (A) and $n = 9$ in (B).

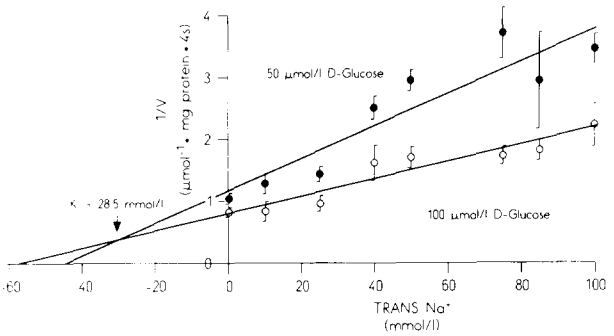

Fig. 9. Effect of *trans* Na^+ on Na^+/D-glucose transport activity. Dixon plot for determination of the inhibitor konstant K_i. Transport was run for 10 s. Mean ± S.E., $n = 7$.

The D-glucose concentration-independent K_i value for *trans* Na^+ inhibition was about 29 mmol/l.

Voltage clamping on oleic acid inhibition of Na^+/D-glucose transport

To preclude an effect of the membrane potential on oleic acid inhibition of Na^+/D-glucose transport experiments were performed under 0 voltage clamped conditions when $K_i = K_{out}$ + valinomycin. Table 4 shows that in 0 clamped BBMV Na^+/D-glucose transport was significantly decreased. However, more importantly 0-clamping did not abolish the inhibition of Na^+/D-glucose transport by oleic acid. Per cent transport inhibition in clamped and non clamped BBMV was in the same range and not significantly different.

H^+-clamping in oleic acid inhibition of Na^+/D-glucose transport

When oleic acid increases Na^+-transport by effecting Na^+/H^+ – exchange the initial outside – inside H^+-equilibrium would be changed. The possible role of a H^+-gradient in oleic acid inhibition of Na^+/D-glucose transport was investigated by using the protonophore FCCP. As can be seen in Table 4 the per cent inhibition of Na^+/D-glucose transport by oleic acid was the same in presence and absence of FCCP which dissipates a possible H^+-gradient. The same result was obtained when FCCP was added to the 0 voltage clamping assay (not shown).

Table 4
Effect of 0 voltage clamping a K^+ diffusion potential with valinomycin and of the protonophore FCCP on the oleic acid inhibition of Na^+/D-glucose transport. Mean ± S.E. n = number of experiments, * = $P < 0.05$

Ionophore	n	Na^+/D-Glucose transport (nmol/mg protein per 15 s)		
		control	oleic acid	change (per cent)
None	10	584 ± 84	330 ± 60 *	−44 ± 9
Valinomycin	10	198 ± 28	86 ± 20 *	−57 ± 10
None	10	634 ± 34	408 ± 39 *	−36 ± 5
FCCP	10	473 ± 31	316 ± 36 *	−39 ± 4

4. Discussion

In the present study it has been shown that oleic acid inhibited Na^+-dependent D-glucose transport rate in renal BBMV, while Na^+/H^+ exchange rate was increased. For explanation of the inhibitory effect of oleic acid on Na^+/D-glucose transport activity the following hypothesis will be discussed: 1) a change in lipid acyl chain order parameter and/or in lipid acyl chain rotational mobility ('fluidity'), 2) a *trans* Na^+ inhibition.

The role of lipid acyl chain order in Na^+/D-glucose transport is supported by the following arguments: first for both probes DPH and 12-AS respectively, we showed a strong correlation between decrease in r_∞ and decrease of Na^+/D-glucose transport. Therefore, we assume that there might be a causal relationship between Na^+/D-glucose transport and lipid acyl chain order.

A decrease in lipid acyl chain order is associated with a lowered molecular packing of acyl chains and a decreased lateral pressure. The lowered surface pressure would lead to a decrease in membrane thickness. These structural contortions of the membrane may allow the 11 membrane spanning segments of the α-helix subunits of the Na^+/D-glucose transporter [44] to adapt to the changed volume between the lipid acyl chains. This may induce a conformational change of the protein which would lower the affinity for Na^+ and/or D-glucose and/or would inactivate the transporter. Evidence that Na^+ binding to the extracellular face leads to a conformational change of the transporter protein has been already presented [45,46]. According to kinetic models additional conformational shifts are believed to occur upon transport of Na^+ and D-glucose to the inner surface and after debinding of both substrates to expose the binding sites once again to the extracellular surface [47].

Additional evidence for an oleic acid induced conformational change of the Na^+/D-glucose transporter is supported by our results on 'fluidity'. The rotational correlation time ϕ of 12-AS correlates strongly with Na^+/D-glucose transport activity. This means that the probe's rotations become faster because of a more 'fluid' environment. Thus, Na^+/D-glucose transport is inhibited with an increase in lipid acyl chain 'fluidity'. The more 'fluid' environment may allow the α-helix of the Na^+/D-glucose transporter protein to undergo a conformational change.

One further argument favors the hypothesis of a 'fluidity' induced conformational change of the Na^+/

D-glucose transporter protein. When bulk membrane 'fluidity' was measured with a set of *n*-(9-anthroyloxy) fatty acid derivates of different chain length which insert in different graded depths of the membrane, the largest oleic acid induced decrease in fluorescence anisoptropy was achieved with 12-AS and 16-AP. These probes have been shown to be located in the middle of the membrane leaflet [42,43]. This is in agreement with the structural requirements for unsaturated *cis* configured LCFA to induce a decrease in lipid acyl chain order. Maximal effects in lowering DPH fluorescence anisotropy were found with the double bound position at C_6 to C_{14}. It is obvious that a membrane spanning transporter protein will exhibit its highest sensitivity to changes in lipid acyl chain order and 'fluidity' in the middle of the membrane.

Obviously the 'fluidity' hypothesis is not fully supported by our kinetic studies with D-glucose. As shown, oleic acid induced an almost twofold increase in the apparent glucose K_m of the Na^+/D-glucose transporter protein while the apparent V_{max} value was only slightly changed. However, as can be exspected, pure 'fluidization' would allow faster oscillations of a transporter protein, resulting in an increase in V_{max} but not of K_m. This is what has been really found for the Na^+-dependent transporter of hexose [5,15] and prolin [17] and also for the Na^+/H^+-exchanger [11], when benzylalcohol or hexanol were used as 'fluidizers'. Obviously, in case of oleic acid a substance specific effect, e.g., a direct interaction with the protein seems to be involved.

Another mechanism by which Na^+/D-glucose transport could be inhibited is an increased Na^+/H^+-exchange. In the literature, this hypothesis was mainly based on two observations: the increase of Na^+/H^+-exchange by the membrane 'fluidizing' agent and the loss of the 'fluidity' effect on Na^+/D-glucose transport in Na^+ equilibrium [2,4,14]. In this study the 'Na^+ gradient collapse theory' has been reinvestigated.

We found that oleic acid facilitated Na^+/H^+-exchange. Under similar conditions as used for Na^+/D-glucose transport (pH equilibrium in presence of 0.1 mmol/l glucose) and at a Na^+ o > i gradient of 50 mmol/l, the Na^+ transport rate of control BBMV was approx. 60 nmol/mg protein per 10 s and increased to approx. 120 nmol/mg protein per 10 s in oleic acid-treated BBMV. With a Na^+ gradient of 100 mmol/l as was used in our D-glucose transport studies, the Na^+ transport rate might have been doubled. Similar Na^+ transport rates of about 30–60 nmol/mg protein per 30 s have been reported for intestinal BBMV [14,48].

The measured Na^+-transport rate means, considering an intravesicular volume of 1.4 μl/mg protein, the intravesicular concentration of Na^+ increases from 42 to 84 mmol/l upon oleic acid addition, i.e., exceeds the K_i for *trans* Na^+ inhibition of D-glucose transport. This suggest the increase in Na^+ transport might be responsible for the inhibition.

However, two contradictory arguments should be considered. It has been reported that *trans* Na^+ reduced the V_{max} but not the K_m of Na^+/D-glucose transport [49,50], whereas oleic acid produced an increase in apparent K_m for glucose with no significant change of the apparent V_{max}. Furthermore it has been shown that Na^+ *trans* inhibition of Na^+-D-glucose transport is highly dependent on the presence of a membrane potential. In the absence of a potential *trans* Na^+ severely inhibits D-glucose influx, but this effect is largely overcome when a potential is present [50]. In contrast we found that the per cent inhibition of Na^+/D-glucose transport was not changed by 0 voltage clamping.

Taken together it may be allowed to conclude that the inhibition of Na^+/D-glucose transport by oleic acid cannot be completely explained by a change in membrane lipid fluidity and *trans* Na^+ inhibition. Future studies will show whether additional mechanisms, e.g., a direct fatty acid-protein interaction or a change in membrane surface charge are also involved in the oleic acid inhibition of Na^+-dependent D-glucose transport. The well known strong binding of LCFA to the BBMV fatty acid binding protein [51] might be taken as indirect evidence for a possible direct interaction of LCFA with the Na^+/D-glucose transporter protein.

5. References

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