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Relation between Ca^{2+} uptake and fluidity of brush-border membranes isolated from rabbit small intestine and incubated with fatty acids and methyl oleate

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The rate of incorporation of oleic acid into isolated brush-border membranes was found to be considerably faster than methyl oleate incorporation under similar experimental conditions. The effects of fatty acids and methyl oleate incorporation on Ca^{2+} uptake and fluidity were monitored. Whereas treatment with 0.01–0.05 mM oleic acid corresponding to incorporations smaller than 90 nmol/mg protein enhanced Ca^{2+} transport, exposures to higher concentrations of this fatty acid corresponding to incorporations larger than 150 nmol/mg protein, decreased uptake of this cation. On the other hand, treatment with 0.01–0.2 mM methyl oleate corresponding to incorporations of up to 220 nmol/mg protein had only a stimulatory effect on the Ca^{2+} uptake. Oleic acid, linoleic acid and methyl oleate decreased the fluorescence anisotropy of membranes labelled with diphenylhexatriene in a dose-dependent manner. In contrast, palmitic acid had little or no effect on the diphenylhexatriene-reportable order of the membrane within the range of concentrations used. Monitored as a function of temperature, the anisotropy values showed a gradual melting for both the control and lipid-treated membranes. The results support the concept that saturated and *cis*-unsaturated fatty acids dissolve in different lipid domains and this in itself appears to be an important factor defining whether the biological function of the membrane is affected by the uptake. Incorporation of *cis*-unsaturated fatty acids in domains harboring the Ca^{2+} uptake process increases Ca^{2+} uptake in concert with increased diphenylhexatriene-monitored fluidity. However, when concentrations of such fatty acids in these domains become sufficiently great, the presence of a largely increased number of free carboxyl groups at the membrane surface causes inhibition of Ca^{2+} uptake.

Introduction

Previous reports have indicated that brush-border membranes isolated from rabbit small intestine can incorporate a variety of lipids in vitro including phospholipids, fatty acids and cholesterol [1–4]. Compositional changes brought about by such incorporations would be expected to change some of the biological properties of the membranes. Accordingly it was shown that the rate of Ca^{2+} uptake was stimulated by treatment of the membranes with low concentrations of *cis*-unsaturated fatty acids (0.05 mM) as well as with various concentrations of caprylic acid (0.10–3.00 mM) and inhibited by treatment with higher concentrations of *cis*-unsaturated fatty acids (0.10–0.60 mM). Saturated fatty acids had no marked effects on Ca^{2+} uptake and glucose and fructose

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transports were unaffected by any of these incorporations [5].

The dual effects of unsaturated fatty acids on Ca^{2+} uptake could not be easily explained although it was suggested that the extent of fluidization of the membrane may be an important factor in determining whether stimulation or inhibition ensues. In the present report we compare the effects of fatty acids and methyl oleate on the fluidity of the membrane as determined by changes in anisotropy using membrane preparations labelled with diphenylhexatriene (DPH). We also compare the effects of oleic acid and methyl oleate on the rate of Ca^{2+} uptake.

Materials and Methods

[1-^{14}C]Oleic acid and tri[1-^{14}C]oleoyl glycerol were purchased from New England Nuclear Corp. (Boston) and diluted to required specific activity. Methyl [1-^{14}C]oleate was formed by alkaline methanolysis of tri[^{14}C]oleoylglycerol [6] and purified by thin-layer chromatography on silica gel G plates prepared with 0.4 M boric acid using petroleum ether (b.p. 60–80 °C)/ethyl ether/acetic acid (90 : 10 : 1, v/v) as solvent [7].

Uptake of fatty acids and methyl oleate. Unlabelled or (1-^{14}C)-labelled fatty acids as well as methyl [1-^{14}C]oleate were dissolved together with sodium taurocholate in chloroform/ethanol (1 : 1, v/v). The solvent was removed by evaporation in vacuo and the residue was suspended in 10 mM Hepes-Tris buffer (pH 7.5), containing 100 mM mannitol. After shaking at room temperature for 15 min, the suspension was sonicated twice for 2 min with the small probe of an Ultrasonics sonicator at a setting of 5. In the case of palmitic acid, the large probe was used instead at a setting of 11. Suspensions that were not completely clear were clarified by centrifugation at 10^7 $g \cdot$ min.

The term, uptake, used in the present report is meant to signify an intercalation of fatty acids within the core of the membrane as opposed to adsorption or loose association at the surface of the membrane. The evidence supporting this was presented in previous reports [4,5] although the possibility of some adsorption cannot be completely precluded. Lipid extraction of the membranes followed by thin-layer chromatographic analyses of the extract indicated that no significant conversion to more complex lipids occurred and essentially all of the incorporated lipid was recovered as free fatty acid.

Brush-border membranes were prepared [8] and checked for purity by electron microscopy and by assay of marker enzymes [9]. For lipid uptake studies, mixtures consisting of membranes (300 μg protein/ml) and various concentrations of micellized fatty acid or methyloleate, as specified in the text, were incubated at 25 °C for 10 min in the case of fatty acids and up to 4 h in the case of methyl oleate. The final taurocholate concentration was 7.2 mM in all cases except for palmitate which required 10 mM bile salt. After the incubation, the membranes were centrifugated at 40 000 $\times g$ for 30 min and the pellet was washed with 1 incubation volume of Hepes-Tris, mannitol buffer. The washed pellet was either counted to estimate the amount of lipid incorporated [5] or resuspended in buffer for further experimentation involving measurements of Ca^{2+} uptake or of fluorescence anisotropy. Protein concentrations were determined by the method of Lowry et al. [10].

Ca^{2+} uptake. The procedure followed was that of Miller and Bronner [11]. Briefly, the uptake was initiated by addition of 10 μl of brush-border membrane vesicles (30 μg protein) to 25 μl of $^{45}CaCl_2$, 0.36 mM final concentration. Incubations were at 25 °C and terminated after 5 min by addition of 40 vol. of ice-cold stop solution containing 10 mM Hepes/Tris buffer (pH 7.5), 5 mM EDTA, 20 μM $LaCl_3$ and 100 mM mannitol. The suspension was then filtered through a 0.45 μm nitrocellulose filter which was then washed twice with 2.5 ml of ice-cold stop solution, air-dried and transferred to counting vials containing PCS (Amersham International)/toluene (1 : 1, v/v) and counted. The rate of uptake under these conditions was linear for at least 5 min [5] and reflected a transport process similar to one described earlier [11].

Spectroscopic measurements. To 2.5 ml of brush-border membranes, (0.16 mg protein/ml) 10 μl of $1 \cdot 10^{-4}$ M diphenylhexatriene in tetrahydrofuran (spectroscopic grade) were added to give a probe: phosphoacylglycerol ratio of 1 : 100. After thorough mixing with a Vortex shaker and incubation at room temperature for 30 min, at which

time little or no odor of tetrahydrofuran could be detected, the suspension was transferred to a 1 cm quartz cell in which mixing was continued by means of a small teflon stir bar. Steady-state anisotropy measurements were made with a Perkin-Elmer spectrofluorimeter, model MPF-44A equipped with a DCSU-2 corrected spectra unit and interfaced with an analog-to-digital converter and a Commodore PET computer. The sample temperature was increased from 0°C at a rate of approx. 1 K/min. Excitation and emission wavelengths were 350 nm and 430 nm and excitation and emission bandpasses were 5 nm and 10 nm, respectively. Corrections for background fluorescence and light scattering were made with blanks containing brush-border membranes and no probe.

Absorbance measurements were conducted with a Varian, Model Cary-219 double beam spectrophotometer equipped with temperature control.

Anisotropy (r) was calculated from the fluorescence intensities measured parallel and perpendicular to the plane of the light of excitation according to the following equation:

$$r = \frac{I_{\parallel} - I_{\perp}(G)}{I_{\parallel} + 2I_{\perp}(G)}$$

where $g = HV/HH$, a correction factor for the anisotropy of the instrument [12].

Total lipids were extracted from microvillus membranes by the method of Bligh and Dyer [13]. For preparation of the sonicated dispersions of lipids (liposomes), the dried, extracted lipid was suspended in 100 mM mannitol 10 mM Hepes-Tris buffer (pH 7.5) to a final concentration corresponding to the studies of the intact vesicles. The mixture was sonicated twice with the small probe of an Ultrasonics sonicator at a setting of 5 for 2 min under N_2 at 25°C. The resulting solutions were clear and were used as such for anisotropy studies.

Results

Results illustrated in Fig. 1 indicate that the incorporation of oleic acid into brush-border vesicles is very rapid, equilibrium being attained immediately. As reported earlier, attempts to reduce this rate by modifying the incubation conditions and sample processing conditions within practical limits were unsuccessful [4]. On the other hand, the incorporation of methyl oleate proceeded at a much slower rate equilibrium being attained after 2 h. It can be noticed that even with methyl oleate, incorporation continues to some extent during processing which extends over a 30 min period corresponding to the time required to pellet the membranes by centrifugation. Some incorporation is consequently seen at zero time.

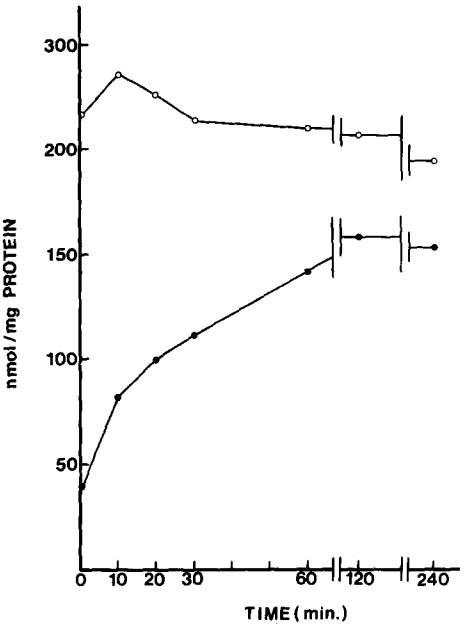

Fig. 1. Time-course of oleic acid (○) and methyl oleate (●) incorporation into brush-border membranes. The incubation mixture contained in 1 ml, 0.3 mg membrane protein, 7.2 mM taurocholate, 0.10–0.11 mM lipid, 100 mM mannitol and 10 mM Hepes-Tris buffer (pH 7.5). Incubations were maintained at 25°C for the times indicated. The values are averages of 3–6 determinations giving variations < 3% and are representative of several other experiments performed under similar conditions with different animals.

Results shown in Table I compare the effects of increasing concentrations of oleic acid and methyl oleate on the rate of uptake of Ca^{2+}. Whereas oleic acid stimulated at lower and inhibited at higher concentrations, the methyl ester analogue was stimulatory over the entire range of concentrations tried. Linoleic acid was shown previously to have the same dual effects as oleic acid whereas palmitic acid had no marked effect [5].

It has been reported that the anisotropy of

TABLE I

EFFECT OF OLEIC ACID AND METHYL OLEATE ON Ca^{2+} UPTAKE

The results represent the mean ± S.E. from 6 to 20 determinations with membranes from 3 to 5 rabbits.

Lipid	Lipid uptake (nmol/mg protein)	Rate of Ca^{2+} uptake (percent of control) [a]
Oleic acid [b]		
0.010 mM	18 ± 1	113 ± 10
0.025 mM	48 ± 0	117 ± 7
0.050 mM	89 ± 4	137 ± 11
0.100 mM	148 ± 3	65 ± 8
0.200 mM	342 ± 17	48 ± 8
Methyloleate [c]		
0.010 mM	14 ± 1	118 ± 7
0.050 mM	68 ± 4	124 ± 8
0.090 mM	101 ± 4	133 ± 9
0.120 mM	119 ± 8	143 ± 7
0.180 mM	182 ± 10	156 ± 10
0.180 mM [d]	220 ± 21	207 ± 29

[a] The control uptake was 7.5 ± 1.5 nmol/mg protein per 5 min.

[b] Values for oleic acid-treated membranes were reported previously [5].

[c] All methyloleate values represent incubations of 1 h with membranes except for (d) in which case incubations were for 4 h.

diphenylhexatriene in membrane preparations was dependent on the membrane concentration [14,15]. This was due to a depolarization of the light caused by scattering in such suspensions. In order to avoid such an artifact we monitored the anisotropy of both the control and treated vesicles at different concentrations of vesicles, as suggested by Teale [14] and Lentz and co-workers [15] and observed this scattering effect. We found that a vesicle concentration corresponding to 0.16 mg protein/ml gave an anisotropy reading which was close to the anisotropy value at infinite dilution (results not shown) and this concentration was used in the experiments that follow.

Results illustrated in Fig. 2 show that treatment of membranes with low and higher concentrations of oleic acid decreased the fluorescence anisotropy in a dose-related manner and these differences became more apparent above 15°C. The results for linoleic acid (Fig. 3 show that for low incorporation of this lipid there is a slight decrease in the anisotropy while for high levels of incorporation (1074 nmol/mg protein) the anisotropy was markedly reduced. Since these fatty acids at low and high concentrations caused stimulation and inhibition of the Ca^{2+} transport, respectively, no

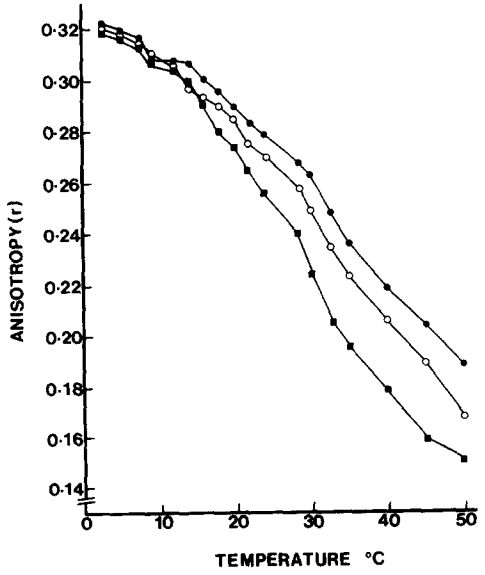

Fig. 2. The effect of oleic acid on anisotropy (●) control, no oleic acid incorporated; (○) 89 nmol (■) 341 nmol oleic acid incorporated/mg protein. The samples contained 0.16 mg of brush-border membrane protein.

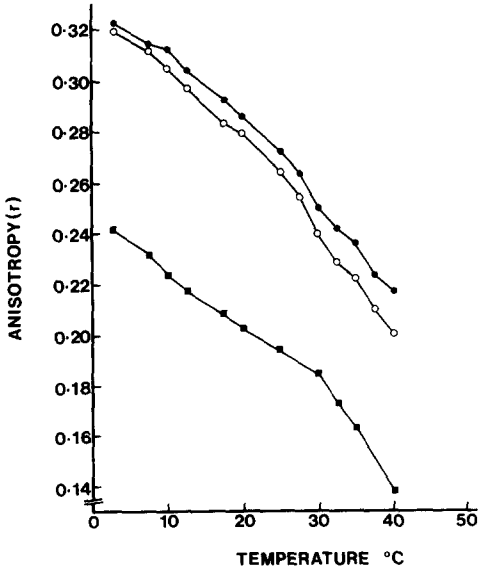

Fig. 3. The effect of linoleic acid on anisotropy (●) control, no linoleic acid incorporated (○) 50 nmol, (■) 1074 nmol linoleic acid incorporated/mg protein. The samples contained 0.16 mg of brush-border membrane protein.

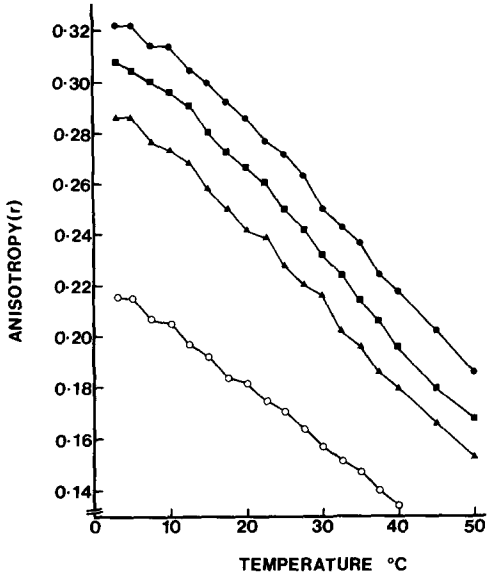

Fig. 4. The effect of methyl oleate on anisotropy (●) control, no methyl oleaete incorporated; (■) 68 nmol, (▲) 101 nmol, (○) 220 nmol methyl oleate incorporated/mg protein. The samples contained 0.16 mg of brush-border membrane protein.

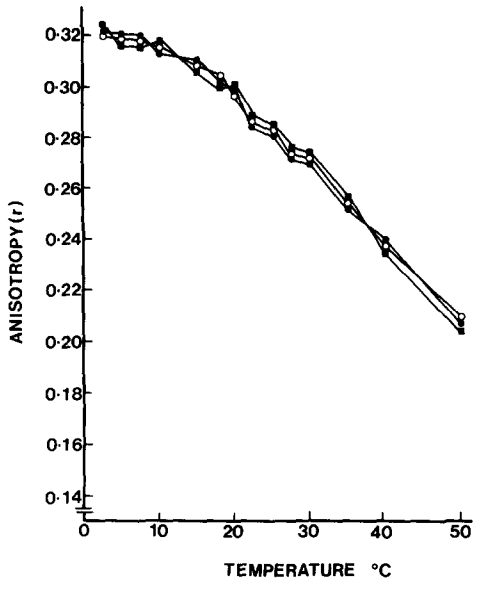

Fig. 5. Effect of palmitic acid on anisotropy (●) control, no palmitic acid incorporated. (○) 153 nmol, (■) 275 nmol palmitic acid incorporated/mg protein. The samples contained 0.16 mg of brush-border membrane protein.

simple relationship between fluidization and transport activation could be found. It was suggested previously that the inhibition seen at high levels of *cis*-unsaturated fatty acid might result from an excess of fluidity which might lead to disorganization of the transport process. However, results in Fig. 4 do not support such a conclusion. Incorporations of 79–220 nmol/mg protein of methyl oleate decreased the anisotropy in a dose-dependent manner yet with this lipid, only a stimulatory effect on Ca^{2+} uptake was seen. By comparison, incorporations of 340 nmol/mg protein of oleic acid inhibited Ca^{2+} uptake (Table I) while the anisotropy of diphenylhexatriene was greater than that found when 220 nmol/mg protein of methyl oleate was incorporated.

It must be noted that the effect on the fluorescence anisotropy of diphenylhexatriene in brush-border membrane vesicles treated with methyl oleate is markedly different from those treated with oleic acid in the same concentration range. As successive amounts of methyl oleate were incorporated into the vesicles the entire anisotropy-temperature plot was shifted to lower values. For example, when 220 nmol/mg protein of methyl oleate was incorporated the anisotropy at 25°C was 0.17 compared with the value of the control sample which was 0.27. When 341 nmol/mg protein of oleic acid was taken up, the anisotropy

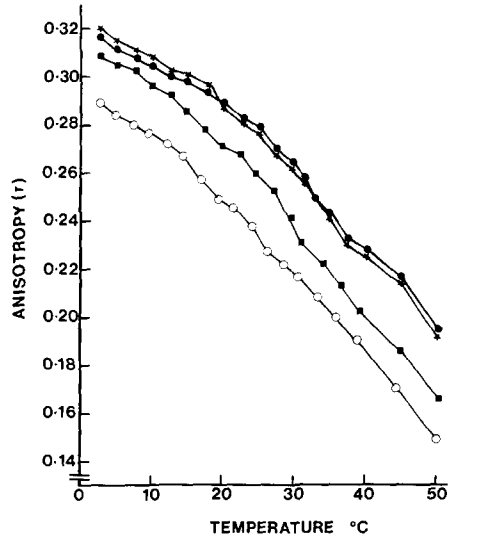

Fig. 6. The lipid extracts of fatty acid-treated brush-border membrane vesicles. (●) control; (*) 153 nmol palmitic acid; (■) 89 nmol oleic acid; (○) 341 nmol oleic acid. The lipid concentrations of the extracts are identical to those used for intact brush-border membrane vesicles.

value was 0.255 at 25 °C. The effect on the fluorescence anisotropy was greater for methyloleate even though less was incorporated than for the oleic acid-treated samples.

Treatment of brush-border membranes with palmitic acid had no significant effect on the anisotropy (Fig. 5). In all cases, the decrease in anisotropy as a function of temperature indicated gradual melting and no sharp transition temperatures.

The results shown in Fig. 6 show a different behavior for the lipids from oleic acid-treated vesicles than was observed for the corresponding intact vesicles. The curve is shifted to lower values over the entire temperature range in the lipid extracts. This contrasts with the intact brush-border membrane vesicles where differences in the anisotropy of the oleic acid-treated vesicles were readily observed only at temperatures greater than 15 °C. The extracts from palmitic acid-treated vesicles gave results similar to those from control samples. Generally, the anisotropy values were lower for the lipid extracts compared to the intact membrane vesicles at temperatures below 30 °C.

Discussion

The reason for the difference in rates of uptake of oleic acid and its methyl ester is not certain. This difference could be related to the negative charge intensity and/or to the size of the lipid-bile salt micelles both of which could affect possible interactions of these aggregates with the membrane. The observation could also be due to differences in concentration of these two lipids in the monomer phase. This point requires further investigation.

It is difficult at this point to provide a fully satisfactory rationalization of the differences in the anisotropy results observed for oleic acid and methyl oleate-treated membranes. The presence of the carboxyl group in the *cis*-unsaturated fatty acids could favor interactions with endogenous membrane constituents such as protein for example which would not occur with the methyl ester derivatives of these fatty acids. Such interactions could restrict the extent of mixing with endogenous lipids and limit the effect of acyl chain unsaturation. The effect of unsaturation would become apparent, however, when relatively high levels of incorporation are attained or when the temperature of the membrane becomes sufficiently elevated.

The results obtained with lipid extracts of the brush-border membranes suggests that the proteins influence the membrane fluidity as has been demonstrated by others [16,17]. This is especially evident with oleic acid-treated membranes the extracts of which display decreased anisotropy even throughout the lower temperature range. These effects of protein become apparent, however, only at temperatures well below physiological.

The present study indicates that while *cis*-unsaturated fatty acids affect the fluidity of the membranes as reported by diphenylhexatriene fluorescence, saturated fatty acids apparently had no effect when similar amounts had been incorporated into the membrane. Our findings are similar to those of Klausner et al. [18], who making use of diphenylhexatriene and 8-anilino-1-naphthalene sulfonic acid as fluorescent probes showed that the membranes of mouse lymphocytes and baby hamster kidney cells contained lipid domains of different fluidities. Saturated and *trans*-unsaturated fatty acids were taken up by the less fluid domains without apparent change in order of the interior apolar regions and consequently diphenylhexatriene fluorescence polarization was unaffected. *Cis*-unsaturated fatty acids on the other hand were taken up by more fluid domains where they caused further disruption of acyl chain packing and a lowering of the diphenylhexatriene fluorescence polarization.

The Ca^{2+} transport channels must in some way interact with such fluid domains since Ca^{2+} transport responds to uptake of *cis*-unsaturated fatty acids as well as to their CoA and methyl ester derivatives [19,20]. Excess fluidization, in response to large incorporations of *cis*-unsaturated fatty acid, cannot be in itself the cause of inhibition of Ca^{2+} transport since higher levels of methyl oleate uptake would have exerted a similar inhibitory effect. One must suppose therefore that the accumulation of a large number of free carboxyl groups in the vicinity of the Ca^{2+} channels is in some manner responsible for the inhibitory effects of oleic and linoleic acids at higher levels of incorporation. The binding of Ca^{2+} to these carboxyl

groups at the exterior of the membrane would compete with Ca^{2+} transport through the channels. Such superficially bound divalent cation would be removed by the wash procedures used in our method for studying Ca^{2+} transport [11] and consequently decreased Ca^{2+} uptake values would be seen. The complete lack of, or relatively weak, inhibitory effect of saturated fatty acids which have been reported to be much more effective in binding Ca^{2+} than *cis*-unsaturated fatty acids, either alone in solution or following uptake by sarcoplasmic reticulum [21–23], could be explained on the basis of their being incorporated in less fluid areas away from the Ca^{2+} channel sites. The presence of free carboxyl groups could also effect conformational changes in the channel proteins or modify charged group relationships across the channel which would in turn alter transport ability. The stimulatory effect of lower levels of *cis*-unsaturated fatty acids could be explained on the basis of increased fluidity at the temperature prevailing during the Ca^{2+} uptake studies since methyl oleate causes the same enhancement of uptake. It could be argued, however, that the stimulation observed at low levels of unsaturated fatty acid is due to some extent to an increase in free calcium at the transport site resulting from the calcium buffer action of carboxyl groups. These points require further clarification and in this respect it would be of interest to test the effect of other unsaturated lipids such as dioleoylphosphatidylcholine, dioleolylphosphatidic acid, alkenylamines and alkenols on Ca^{2+} transport.

The luminal membrane of intestinal cells is constantly exposed to exogenous lipids derived from the diet. The uptake of these lipids modifies the composition of the membrane and this in turn would affect its structure relationships. Some of the possible changes in structure and function are described in the present study which reveals that oleic acid and methyl oleate increases the fluidity of the membrane in a dose-dependent manner while affecting transport of Ca^{2+}. The effects on Ca^{2+} transport are complex however and do not relate only to general physical effects on the membrane but also to specific differences in the molecular structure of the incorporated lipids. Purification of the transporter proteins and their reconstitution into liposomes could shed additional light on the overall effects of lipids on Ca^{2+} transport as it occurs in brush-border membranes.

Acknowledgements

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