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THE ROLE OF FATTY ACIDS IN THE REGULATION OF BRUSH-BORDER Ca^{2+} TRANSPORT

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The role of the fatty-acid composition of the intestinal brush-border membrane in the control of transmembrane Ca^{2+} transport was examined by in vitro acylation of endogenous phospholipids. The initial rate of Ca^{2+} uptake into brush-border membrane vesicles was stimulated 1.6-fold by incubation with 50 μM linoleoyl CoA, which was the most effective CoA ester examined. Oleoyl CoA was also active, but stearoyl CoA, palmitoyl CoA and arachidonyl CoA displayed no activity. The effect of linoleoyl CoA was specific for Ca^{2+} transport; sodium-dependent phosphate uptake was slightly inhibited and the activity of alkaline phosphatase, a brush-border enzyme, was unaffected. Incubation of brush-border vesicles with either stimulatory (oleate) or nonstimulatory (stearate) CoA esters resulted in the incorporation of fatty acid into the four major phospholipid classes, suggesting a fatty-acid specificity of the Ca^{2+} transport phenomenon. These results are consistent with the hypothesis that fatty acids are important elements in the control of brush-border Ca^{2+} transport.

Changes in the lipid composition of the plasma membrane influence the reactions associated with this membrane through alterations in protein-lipid interactions [1]. Physical manipulation of the lipid environment has been reported to affect activities including $(Na^+ + K^+)$-ATPase [2], $(Ca^{2+} + Mg^{2+})$-ATPase [3], adenylate and guanylate cyclase [4,5] and hormone-receptor interactions [6]. Hormonal-stimulation can also induce changes in plasma membrane lipids, and through this mechanism, may affect cellular metabolism. We have studied the role of lipid changes in the action of two steroid hormones: aldosterone and 1,25-dihydroxyvitamin D_3. The former stimulates the synthesis and incorporation of fatty acids into phospholipids of the toad urinary bladder [7]. Inhibition of acetyl CoA carboxylase, the rate-limiting step in fatty acid synthesis, inhibits the change in the fatty-acid composition of the bladder in response to aldosterone and prevents the stimulation of Na^+ transport [8]. 1,25-dihydroxyvitamin D_3 affects both the fatty-acid and phospholipid composition of the intestinal brush-border membrane. In response to this hormone, there is an increase in phosphatidylcholine and in the amount of linoleic and arachidonic acid in this phospholipid fraction, and these changes are correlated with changes in Ca^{2+} transport [9,10].

In this study, we examined the effect of fatty acids on the Ca^{2+} transport properties of intestinal brush-border membranes. Fatty acids were incorporated into membrane phospholipids through their CoA derivatives. Long-chain unsaturated fatty acids stimulated Ca^{2+} uptake, whereas saturated fatty acids were without effect. These results illustrate the role of fatty acids in regulating the rate of Ca^{2+} transport across the intestinal brush-border membrane.

Abbreviation: Mes, 4-morpholineethanesulfonic acid.

Methods

1-day-old, white Leghorn cockerels (Moyer's Chicks, Quakertown, PA) were maintained for 1 week on a vitamin D-deficient diet with a soy protein base (Teklad No. 170245) and then on a vitamin D-deficient diet containing lipid-extracted casein as the protein source (Teklad TD81011). Chicks were killed between 3 and 5 weeks of age. All animals were fasted overnight, and those receiving $1,25(OH)_2D_3$ were injected intraperitoneally with 1 μg in 100 μl propylene glycol 16 h prior to being killed. Brush-border membranes were isolated as previously described [9] and assayed the same day. All experiments contained at least four chicks per group.

The effect of fatty acid incorporation on the transport properties of brush-border membrane vesicles was examined by incubating vesicles at 0.5 mg/ml for 20 min at 34°C with fatty acyl CoA in 75 mM Tris/75 mM Mes/75 mM KCl/0.1 mM $CaCl_2$/0.5 mM $MgCl_2$ (pH 7.4). At the end of this incubation period, vesicles were washed with 1 mg/ml fatty-acid-poor bovine serum albumin, centrifuged at 20000 × g for 30 min and resuspended at 0.5 mg/ml in incubation buffer. Ca^{2+} and phosphate uptakes were measured at 34°C as previously described [11,12]. The alkaline phosphatase activity of the membrane vesicles was measured using p-nitrophenyl phosphate as substrate by a previously described method [11].

Incorporation of fatty acids into brush-border phospholipids was examined using radiolabeled fatty acyl CoA. Incubations were performed as described above for transport, except that 50 μM oleoyl or stearoyl CoA containing 0.2 μCi [1-^{14}C]oleoyl or [1-^{14}C]stearoyl CoA, respectively, was employed. After washing with fatty-acid-poor bovine serum albumin and centrifuging, lipids were extracted [13]. Phospholipids were separated on Redi-coat H plates using a solvent system of chloroform/methanol/acetic acid/water (50 : 30 : 8 : 4) [14]. Free fatty acids were separated from neutral lipids on Redi-coat G plates with a solvent system of hexane/diethylether/acetic acid (70 : 30 : 1) [15]. Spots were localized with iodine vapor, scraped into scintillation vials and counted.

Materials

Fatty acyl CoA's were obtained from Sigma (St. Louis, MO, U.S.A.). [1-^{14}C]Oleoyl CoA and [1-^{14}C]stearoyl CoA were from Amersham (Arlington Heights, IL, U.S.A.). Redi-coat H and G plates were from Supelco (Bellefonte, PA, U.S.A.). $1,25(OH)_2D_3$ was the generous gift of Dr. W.E. Scott of Hoffmann-LaRoche (Nutley, NJ, U.S.A.)

Results

The role of fatty acids in the regulation of Ca^{2+} transport was examined by incubating fatty acyl CoA's with brush-border membrane vesicles isolated from vitamin D-deficient chicks. At 50 μM, linoleoyl CoA increased the initial rate (1 min time-point) of Ca^{2+} uptake from 3.86 to 6.28 nmol/min per mg protein ($P < 0.005$; Fig. 1). The increased uptake was sustained up to 20 min, but not beyond. When brush-border vesicles from $1,25(OH)_2D_3$-treated chicks were incubated with 50 μM linoleoyl CoA, the same rate of transport

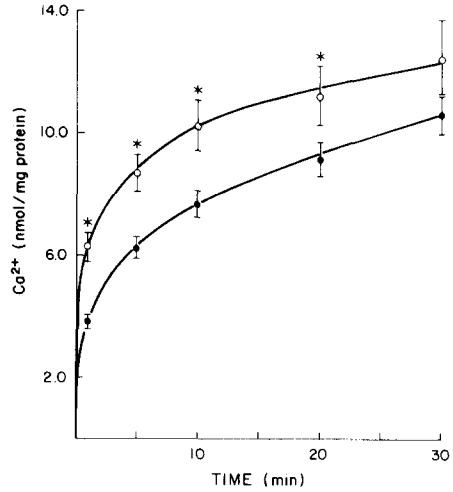

Fig. 1. Stimulation of Ca^{2+} uptake into brush-border membrane vesicles by linoleoyl CoA. Vesicles from vitamin D-deplete chicks were incubated with 50 μM linoleoyl CoA for 20 min at 34°C. The reaction was terminated with 1 mg/ml fatty-acid-poor bovine serum albumin followed by centrifugation at 20000 × g. The vesicles were resuspended at 0.5 mg/ml and Ca^{2+} uptake was measured. Values represent mean ± S.E. of seven separate experiments employing separate membrane preparations. (*) $P < 0.05$ compared to control; (●——●) control; (○——○) 50 μM linoleoyl CoA.

was observed as for vesicles from vitamin D-deficient chicks incubated under identical conditions. As shown in Table I, Ca^{2+} uptake at 1 and 5 min in vitamin D-deplete vesicles incubated with linoleoyl CoA was 6.28 and 8.70 nmol/mg protein, respectively, as compared to 6.24 and 8.45 nmol/mg protein for vitamin D-treated vesicles incubated with linoleoyl CoA. These results suggest a saturability to the linoleoyl CoA effect.

As shown in Table II, the rate of Ca^{2+} transport was dependent on the concentration of linoleoyl CoA. At 25 μM, linoleoyl CoA stimulated Ca^{2+} uptake into vesicles 44% (5.55 vs. 3.86 nmol/min per mg; $P < 0.05$) relative to 63% at 50 μM. It was not possible to test concentrations much higher than 50–75 μM because of detergent effects of these acyl CoA molecules [16].

The fatty-acid specificity of the transport response was examined through the use of various other CoA derivatives (Table II). The fatty acyl CoA's were divided into two groups: saturated and unsaturated. The former group was composed of stearoyl CoA (18 : 0) and palmitoyl CoA (16 : 0), the latter group linoleoyl CoA (18 : 2), arachidonyl CoA (20 : 4) and oleoyl CoA (18 : 1). Linoleoyl CoA was clearly the most effective modulator of Ca^{2+} transport. Oleoyl CoA was also active, albeit less so than linoleoyl CoA. At 50 μM, oleoyl CoA stimulated Ca^{2+} uptake 40% (5.42 vs. 3.86 nmol/min per mg; $P < 0.025$), which is comparable to that observed with 25 μM linoleoyl CoA. Somewhat surprising was the inactivity of arachidonyl CoA. This, however, may be due to the marked instability of this compound. The saturated fatty acyl derivatives were also inactive. At 50 μM, neither stearoyl CoA nor palmitoyl CoA had a significant effect on the rate of Ca^{2+} uptake ($P > 0.05$). Coenzyme A or methyl linoleate at 50 μM were also without effect (data not shown).

The incorporation of the fatty-acid moiety of the CoA derivative into the various phospholipid fractions and the liberation of free fatty acids were examined by measuring the rate of [1-^{14}C]oleoyl CoA and [1-^{14}C]stearoyl CoA. These two CoA's were chosen because one (oleoyl CoA) stimulated Ca^{2+} transport, while the other did not. Labeled oleoyl CoA was employed instead of linoleoyl CoA because of the unavailability of the latter and the difficulty in synthesizing it in a biologically active form [17,18]. As shown in Table III, oleic

TABLE I

EFFECT OF LINOLEOYL CoA ON THE INITIAL RATE OF Ca^{2+} UPTAKE INTO BRUSH-BORDER VESICLES FROM VITAMIN D-DEPLETE AND 1,25$(OH)_2D_3$-TREATED CHICKS

Values (nmol Ca^{2+}/mg protein) are means ± S.E. Numbers in parentheses indicate number of separate experiments. − D, vitamin D-deplete; + D, vitamin D-treated.

	Addition	Ca^{2+} uptake	
		1 min	5 min
− D	none	3.86 ± 0.19	6.26 ± 0.36 (7)
+ D	none	4.77 ± 0.19	7.25 ± 0.37 (6)
− D	linoleoyl CoA	6.28 ± 0.47	8.70 ± 0.57 (8)
+ D	linoleoyl CoA	6.24 ± 0.39	8.45 ± 0.48 (5)

TABLE II

EFFECT OF CoA DERIVATIVES ON THE INITIAL RATE OF Ca^{2+} UPTAKE

Values (nmol Ca^{2+}/min per mg protein) are means ± S.E. Numbers in parentheses indicate number of separate experiments.

Addition	Concentration (μM)	Ca^{2+} uptake	Stimulation (%)
None	–	3.86 ± 0.19 (5)	–
Linoleoyl CoA	25	5.55 ± 0.51 (5)	44 [a]
Linoleoyl CoA	50	6.28 ± 0.47 (6)	63 [a]
Arachidonyl CoA	50	4.35 ± 0.72 (3)	13
Oleoyl CoA	50	5.42 ± 0.74 (3)	40 [a]
Stearoyl CoA	50	4.58 ± 0.39 (6)	19
Palmitoyl CoA	50	4.25 ± 0.27 (6)	10

[a] $P < 0.05$.

TABLE III

INCORPORATION OF [^{14}C]OLEOYL CoA AND [^{14}C]STEAROYL CoA INTO BRUSH-BORDER MEMBRANES

Values are mean ± S.E. of three separate experiments.

Lipid class	nmol oleoate/ mg protein	nmol stearate/ mg protein
Phosphatidylcholine	1.30 ± 0.11	4.02 ± 0.15
Phosphatidylserine/inositol	0.68 ± 0.10	1.43 ± 0.20
Phosphatidylethanolamine	1.26 ± 0.19	1.43 ± 0.20
Free fatty acid	3.84 ± 0.88	2.82 ± 0.13

acid was incorporated into phosphatidylcholine and phosphatidylethanolamine equally well (1.30 and 1.26 nmol/mg protein, respectively), but less so into a combined phosphatidylserine/inositol fraction (0.68 nmol/mg protein). Incubation of brush-border membrane vesicles with 50 μM oleoyl CoA resulted in the liberation of 3.84 nmol oleic acid/mg protein. Incubation of brush-border vesicles with 50 μM stearoyl CoA, which had no effect on Ca^{2+} transport, resulted in the incorporation of stearate primarily into phosphatidylcholine (4.02 nmol/mg protein) with lesser, but equimolar, amounts in phosphatidylethanolamine and phosphatidylserine/inositol (1.43 nmol/mg protein). Similar amounts of free fatty acid were generated as during incubation with oleoyl CoA (2.82 vs. 3.84 nmol/mg protein, respectively; $P > 0.05$).

Sodium-dependent phosphate uptake was measured following incubation of brush-border membrane vesicles with linoleoyl CoA to ascertain the specificity of the transport response. The effect of linoleoyl CoA on phosphate uptake could not be measured using the bovine serum albumin wash technique; under these conditions, no overshoot was observed. This was unexpected, since Ca^{2+} uptake was unaffected by the wash. We therefore omitted the wash step when we examined the effect of linoleoyl CoA on phosphate uptake. As shown in Fig. 2, the initial rate of phosphate uptake was slightly reduced following incubation of the vesicles with 50 μM linoleoyl CoA. Neither the magnitude of the overshoot nor the equilibrium value for phosphate uptake was affected, the latter suggesting that the intravesicular volume is unaffected by linoleoyl CoA. Incubation of vesicles with 50 μM stearoyl CoA had no effect on phosphate transport (not shown).

Alkaline phosphatase, a membrane-bound enzyme, was measured following incubation with 50 μM linoleoyl or stearoyl CoA. One would expect that gross alterations in the membrane, for example, detergent-like effects, would be reflected in the activity of membrane-bound proteins. There was no effect of either CoA derivative on the activity of alkaline phosphatase. The rate of hydrolysis of *p*-nitrophenyl phosphate was 20.4 ± 0.1 nmol/min per mg protein for control vesicles as compared to 22.2 ± 0.3 and 21.6 ± 0.2 nmol/min per mg protein for linoleoyl CoA- and stearoyl CoA-treated vesicles, respectively.

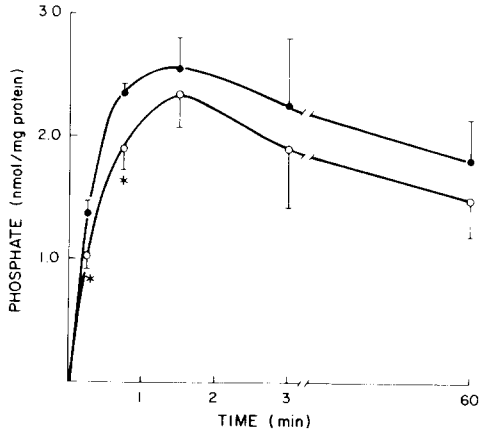

Fig. 2. Sodium-dependent phosphate uptake into vesicles incubated with linoleoyl CoA. Experimental conditions as described in the legend to Fig. 1 with the exception of the bovine serum albumin wash. Values represent mean ± S.E. of three separate experiments employing separate membrane preparations. (*) $P < 0.05$; (●———●) control; (○———○) 50 μM linoleoyl CoA.

Discussion

The present studies demonstrate that incubation of isolated brush-border membrane vesicles with linoleoyl CoA results in the stimulation of the rate of calcium uptake into these vesicles. This is most likely due to the presence of sufficient lysophospholipids in the membrane such that incubation with linoleoyl CoA results in the enzymatic esterification of linoleic acid. The stimulation of calcium transport was specific for the fatty acyl group of the CoA ester. Oleoyl CoA was approx. 50% as effective as linoleoyl CoA, but neither arachidonyl CoA, stearoyl CoA nor palmitoyl CoA induced a significant change in calcium transport. The inactivity of the latter compounds cannot be explained by their lack of incorporation into phospholipid. Stearic acid was incorporated into phosphatidylcholine to a greater extent than was oleic acid. Unfortunately, incorporation of labeled linoleic acid employing [^{14}C]linoleoyl CoA was not carried out because of the unavailability of this substrate, and the difficulty in preparing it in a biologically active form [17,18].

However, in the case of oleoyl CoA, which also stimulated calcium transport, equal quantities of oleic acid were incorporated into phosphatidylcholine phosphatidylethanolamine.

Incubation of brush-border membranes with the CoA derivatives also resulted in the liberation of free fatty acids, which raised the possibility that the stimulation of Ca^{2+} transport was due to a detergent-like effect of the fatty acid. This does not appear to be the case. Incubation with 50 μM methyl linoleate had no effect on the rate of Ca^{2+} uptake. In addition, treatment of vesicles with 50 μM oleoyl CoA followed by a bovine serum albumin wash resulted in the liberation of 3.84 nmol oleic acid/mg protein. If the wash step was omitted, the amount of free oleic acid was 20.7 nmol/mg protein. Thus, the wash was very effective in removing the free fatty acids that were liberated during the incubation. Interestingly, the stimulation of Ca^{2+} uptake was not diminished by the bovine serum albumin wash. Linoleoyl CoA at 50 μM stimulated the initial rate of Ca^{2+} transport 63% under conditions in which free fatty acids were removed by bovine serum albumin treatment and 66% when this step was omitted. Furthermore, the 3.84 nmol/mg oleic acid remaining after the incubation corresponds to a concentration in solution of 7.0 μM. Previous results from our laboratory demonstrated that concentrations of vaccenic acid, a fatty acid identical to oleic acid except for the position of the double bond, up to 120 μM had no effect on the rate of Ca^{2+} uptake into brush-border membrane vesicles [19].

Our interest in the involvement of fatty acids in the Ca^{2+} transport process arose from our work with 1,25-dihydroxyvitamin D_3. This hormone induces at least three distinct functional changes in brush-border membrane vesicles isolated from chick duodenal mucosa: (1) an increase in the rate of uptake of Ca^{2+} into the vesicle [9]; (2) an increase in the rate of Na^+-dependent phosphate uptake into the vesicle [4]; and (3) an increase in the activity of the enzyme alkaline phosphatase [4]. These three changes occur with different time-courses, and appear by several criteria to be independent of one another [12,19,20]. The change in rate of calcium uptake is the earliest change observed, and its time-course of change is similar to the time-course of change in membrane lipid turnover and structure [10].

The role of brush-border membrane lipid environment in the regulation of Ca^{2+} transport has been illustrated by in vitro manipulation [19]. In that study, *cis*- and *trans*-vaccenic acid methyl esters were used to perturb the membrane, most likely by a change in fluidity [21]. These methyl esters are not enzymatically incorporated into phospholipid, but rather simply partition into the bilayer. In the present study, we examined the effect on Ca^{2+} transport of the incorporation of fatty acids into phospholipids. This is similar to the action of 1,25-dihydroxyvitamin D_3 on the duodenal mucosa, which increases the content of linoleic and arachidonic acid in the phosphatidylcholine fraction [2,7]. The present data argue that this is an important event in the stimulation of intestinal Ca^{2+} transport, but does not allow us to discriminate between synthesis of new phospholipid and the deacylation-reacylation of existing phospholipid.

Finally, in terms of specificity, it is again noteworthy that incubation of vesicles with linoleoyl CoA under conditions which led to a significant increase in the rate of calcium uptake into these vesicles, had no effect on the activity of alkaline phosphatase, and only a slight inhibitory effect on Na^+-dependent phosphate uptake, whereas the in vitro administration of $1,25(OH)_2D_3$ causes significant increases in both the activity of this enzyme and the rate of Na^+-dependent phosphate uptake [4]. The present data provide further evidence that these three changes in membrane function are brought about by different biochemical mechanisms. They also provide evidence that the subtle changes in membrane lipid structure can exert specific, rather than nonspecific, effects on membrane function.

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