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VITAMIN D-MEDIATED INTESTINAL CALCIUM TRANSPORT

EFFECTS OF ESSENTIAL FATTY ACID DEFICIENCY AND SPIN LABEL STUDIES OF ENTEROCYTE MEMBRANE LIPID FLUIDITY *

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Vitamin D-3 and its metabolites regulate the transport of calcium across the intestinal epithelial cell via a mechanism which is as yet unknown. The purpose of this study was to evaluate the effect of an essential fatty acid deficiency on vitamin D-stimulated intestinal calcium transport as measured by both in vivo and in vitro techniques. We also describe in this report a procedure for the isolation of chick intestinal epithelial cell brush border and basal lateral membranes and an assessment of the effect of dietary vitamin D on the lipid fluidity of these membranes. An essential fatty acid deficiency in both vitamin D-replete and deficient chicks resulted in a decrease in intestinal mucosal levels of linoleic acid, with a compensatory increase in the levels of the short chain fatty acid, myristic acid, and the unsaturated fatty acids, palmitoleic and eicosatrienoic acids. An essential fatty acid deficiency did not affect the ability of vitamin D-deficient chicks to respond to vitamin D with a 2-fold increase in serum calcium and a 4–5-fold increase in intestinal calcium transport, measured in vivo. However, an essential fatty acid deficiency resulted in an inability of vitamin D to increase calcium flux in vitamin D-deficient chick ileum as measured under in vitro conditions. Dietary vitamin D resulted in no detectable change in the protein composition in either the brush border or basal lateral membranes as evidenced by SDS-polyacrylamide electrophoresis. In addition, vitamin D did not alter the levels of brush border membrane cholesterol or lipid phosphorus (0.27 ± 0.03 and 0.19 ± 0.01 μmol/mg protein, respectively). Brush border and basal lateral membranes were labeled with the 5-nitroxide stearate spin probe I(12,3). The polarity of the environment of the probe in the brush border membranes is much greater than that of the basal lateral membranes. In addition, the lipid environment of the brush border membrane is much less fluid ($S = 0.650$) than that of the basal lateral membrane ($S = 0.583$). The data concerning membrane lipid fluidity is qualitatively similar to fluorescence polarization studies of rat intestinal epithelial cell membranes and confirms the concept that a given cell may contain plasma membrane regions having discrete lipid structures/fluidities. Dietary vitamin D had no detectable effect on the lipid fluidity or polarity in either the brush border or basal lateral membranes. The results do not support a role for an alteration in essential fatty acid composition or gross changes in the lipid fluidity of the brush border or basal lateral membranes as mechanisms by which vitamin D regulates intestinal calcium transport.

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Abbreviations: $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D-3; EGTA, ethyleneglycol bis(β-aminoethyl ether)N,N'-tetraacetic acid; Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid.

Introduction

It is now well established that vitamin D-3 is metabolized to the hormonally active form 1,25-dihydroxyvitamin D-3 ($1,25(OH)_2D_3$) [2]. However, relatively little is known concerning how vitamin D and its metabolites maintain, modify or coordinate the cellular processes which facilitate the primary biological response to vitamin D-3, namely the maintenance of adequate intestinal calcium and phosphate transport [2].

Recently, efforts to elucidate the molecular mechanism of vitamin D-stimulated intestinal calcium transport have concentrated on an evaluation of vitamin D-dependent alterations in the lipid and protein composition of the intestinal epithelial cell brush border membrane. Vitamin D-dependent alterations in intestinal mucosal and brush border lipids have been observed [3–5]. In addition vitamin D treatment has been reported to increase the activity of intestinal phosphatidylcholine acyltransferase [6]; it has also been reported that an essential fatty acid deficiency inhibits the vitamin D-3-mediated increase in calcium uptake as measured by in vitro techniques [7]. It is possible that these lipid perturbations are associated with alterations in the membrane lipid fluidity. The lipid fluidity of biological membranes is known to be an important parameter in regulating a wide variety of membrane-associated functional processes [8,9] including the activities of (Na^+, K^+)-ATPase [10], hormone-stimulated adenylyl cyclase [11–13], glucose transport [14,15] and the ion permeability of the membrane [8,9]. Recent studies have focused on the role of the lipid fluidity of the rat intestinal cell membrane in the expression of enterocyte enzymatic activities. For example, Brasitus and Schachter [16] reported on the basis of experiments employing fluorescence labels, that the activities of basal and prostaglandin E_1-stimulated adenylyl cyclase are particularly sensitive to alterations in the lipid fluidity of the basal lateral membrane.

These observations suggest that vitamin D-dependent alterations in intestinal epithelial cell membrane lipid composition and/or organization could regulate the calcium permeability of the intestinal cell. The feasibility of such a mechanism has been previously demonstrated with use of the polyene antibiotic filipin [17,18]. When filipin is applied in vitro to the mucosal surface of duodenum from vitamin D-deficient chicks there is induced an active transport system for calcium which mimics many characteristics of normal in vivo vitamin D-stimulated intestinal calcium transport. This apparently results from the reorganization of membrane lipids induced by the specific association of filipin with membrane bound cholesterol [19]. Recently it has been demonstrated that the incorporation of *cis*- and *trans*-vaccenic acid into brush border membrane vesicles also mediates specific alterations in the calcium uptake properties of isolated vesicles [20].

In light of these observations, we report here the application of electron spin resonance (ESR) spectroscopy to detect possible vitamin D-dependent alterations in the lipid fluidity of brush border, Golgi and basal lateral membranes from chick intestinal epithelial cells. In addition, we have evaluated the effects of an essential fatty acid deficiency on vitamin D-dependent intestinal calcium transport as measured by both in vivo and in vitro techniques.

Materials and Methods

Animals. White Leghorn cockerels were raised for 3 to 4 weeks on a standard vitamin D-deficient diet [21] or a vitamin D- and essential fatty acid-deficient diet prepared by a modification of the diet described by Hill [22]. Calcium and phosphate levels in the vitamin D- and essential fatty acid-deficient diets were adjusted to the percentages used in the vitamin D-deficient diet, namely 0.6% calcium and 0.4% phosphorus.

Vitamin D-replete chicks received oral doses of 500 I.U. * (32.5 nmol) of vitamin D-3 96, 72, 48 and 24 h prior to sacrifice. Vitamin D-deficient chicks received the dose vehicle (ethanol/1,2-propanediol (1 : 1, v/v)).

Lipid extraction and fatty acid analysis. 1 g of intestinal mucosa was extracted with 40 ml of chloroform/methanol (1 : 2, v/v) essentially by the method of Bligh and Dyer [23]. The organic solvent extract was divided into two phases by the addi-

* One international unit (I.U.) of vitamin D-3 is equivalent to 25 ng or 65 pmol.

tion of one part of 1% NaCl and the chloroform layer was then collected and subsequently dried under nitrogen. The lipid residues were saponified and the fatty acids were converted to methyl esters. These were then analyzed with the use of a Perkin-Elmer F11 gas chromatograph with a flame ionization detector. The fatty acids were tentatively identified essentially as described by Woodford [24].

Calcium transport methods. Calcium absorption in vivo was measured according to the procedure of Coates and Holdsworth [25] as modified by Hibberd and Norman [26]. Calcium flux in vitro was determined, under non-short-circuit conditions, by measuring the $^{45}Ca^{2+}$ transport across an ileal segment mounted in a 'Ussing apparatus', exactly as previously described [17]. Serum calcium was determined with a Perkin-Elmer Model 303 atomic absorption spectrophotometer.

Membrane isolation. Twenty cm duodenal segments (measured immediately distal to the pylorus) from four chicks were utilized for a typical membrane isolation. The duodena were removed, everted and washed thoroughly with ice-cold 0.9% NaCl.

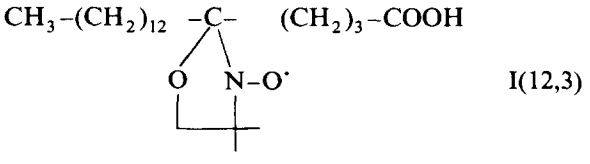
Intestinal epithelial cells were prepared according to a modification of the procedure of Stern et al. [27]. The everted duodenal segments were incubated at 37°C for 15 min in a sodium citrate buffer (1.5 mM KCl, 96 mM NaCl, 27 mM sodium citrate, 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, pH 7.3). They were then placed in 90 mM NaCl, 5 mM KCl, 50 mM phosphate, 1.5 mM EDTA, pH 7.4 and the cells were dissociated from the villi by gentle agitation with a stirring rod for 10 min at 37°C. Intestinal epithelial cells were collected by centrifugation at $900 \times g$ for 5 min and washed twice by resuspension in phosphate-buffered saline followed by centrifugation. The final washed cellular pellet was used as a source of starting tissue for membrane isolation (described below).

Crude membrane fractions were obtained via a differential centrifugation scheme adapted from the procedure of Mircheff and Wright [28]. The procedure involves an initial homogenization (Dounce type homogenizer) of the cells in hypotonic 5 mM EDTA, pH 7.5, with subsequent high speed ($37000 \times g$ for 30 min) centrifugation to yield a particulate and soluble fraction. Crude brush border and basal lateral-Golgi membranes were obtained from the particulate material by a series of sequential homogenizations and low speed centrifugations. The differential centrifugation scheme described by Mircheff and Wright [28] is followed to yield pellets P_3 and P'_4, designated crude brush border and crude basal lateral-Golgi membranes, respectively.

Crude brush border membranes were resuspended in 20 ml of 50 mM $MgCl_2$, 5 mM EGTA, 10 mM Hepes, pH 7.4 and centrifuged at $450 \times g$ for 15 min. Brush border membranes in the resulting brush border-nuclear pellet were further purified by sequential sucrose and glycerol step density gradients according to the procedure of Max et al. [29].

Crude basal lateral-Golgi membranes were homogenized in 15 ml of 0.69 M sorbitol, 0.5 mM EDTA, 5 mM histidine-imidazole, pH 7.5 by 10 strokes in a glass-Teflon homogenizer (1000 rpm). The resulting homogenate was layered on a 30 ml 30–60% linear sorbitol density gradient formed in a Beckman SW-27 centrifuge tube. After equilibrium centrifugation for 18–19 h at 25000 rpm the gradients were fractionated from the top by forcing dense sucrose into the bottom of the tube (all gradient solutions contained 0.5 mM EDTA, 5 mM histidine-imidazole, pH 7.5). The sedimentation position of basal lateral and Golgi membranes was determined by assay of the marker enzymes (Na^+, K^+)-ATPase and galactosyl transferase, respectively. The refractive indices of the gradient fractions containing the peak enzyme activities were pooled. Basal lateral and Golgi membranes in the pooled fractions were collected by centrifugation at $37000 \times g$ for 30 min.

Spin label studies. Membrane fractions were labeled with the spin probe 5-nitroxide stearate (I(12,3)), where nitroxide refers to the 4',4'-dimethyl-*N*-oxyl-oxazolidine ring (Syva Co., Palo Alto, CA).



I(12,3)

The label was dissolved in absolute ethanol

(10^{-3} M) and stored at −70°C in liquid nitrogen storage tubes (Microbiological Associates, Los Angeles, CA). Freshly prepared brush border, basal lateral or Golgi fractions were labeled with I(12,3) at room temperature as described previously by Sauerheber et al. [30]. The ratio of spin label to membrane protein was varied from 2.2 to 14.6 nmol I(12,3)/310 μg membrane protein for the various membrane preparations. ESR spectra were recorded after a 4–5-min period for temperature equilibration of the sample with a Varian E-104A Century Series ESR spectrometer equipped with a Varian variable temperature accessory [31]. The ESR spectra of the I(12,3)-labeled intestinal cell membrane fractions are similar to those reported earlier for other I(12,3)-labeled model and biological membranes [30,31,34,35]. The spectra indicate that the label undergoes rapid anisotropic motion about its long molecular axis in the membrane in an apparently homogenous lipid environment; flexing or bending motions of the probe (i.e. the angular deviation of the hydrocarbon chain away from the preferred orientation perpendicular to the membrane surface) appear to be relatively restricted. No evidence is apparent that the probe incorporates simultaneously into lipid domains of differing fluidities in any of the examined intestinal membrane fractions. The membrane fluidity (or more accurately, the flexibility of the incorporated fatty acid probe) was quantitated by first measuring the outer and inner hyperfine splittings $2T_{\parallel}$ and $2T_{\perp}$ (see Fig. 1 in Sauerheber et al. [30] for I(12,3)-labeled adipocyte ghosts) and then employing the following order parameter expressions [32]:

$$S(T_{\parallel}) = 1/2\left[\frac{3(T_{\parallel} - T_{xx})}{(T_{zz} - T_{xx})} - 1\right] \quad (1)$$

$$S(T_{\perp}) = 1/2\left[\frac{3[(T_{zz} + T_{xx}) - 2T_{\perp}]}{(T_{zz} - T_{xx})} - 1\right] \quad (2)$$

$$S = \frac{(T_{\parallel} - T_{\perp})(a_N)}{(T_{zz} - T_{xx})(a'_N)} \quad (3)$$

T_{xx} and T_{zz} were previously determined by incorporating nitroxide derivatives into host crystals as substitutional impurities: $(T_{xx}, T_{zz}) = (6.1, 32.4G)$ [33]. a'_N and a_N are the isotropic hyperfine coupling constants for the probe in the membrane and crystal state, respectively ($a'_N = 1/3(T_{\parallel} + 2T_{\perp})$ and $a_N = 1/3(T_{zz} + 2T_{xx})$). a'_N is sensitive to the polarity of the membrane environment of the probe [32,34].

The order parameters S, $S(T_{\parallel})$, and $S(T_{\perp})$ were here employed to not only monitor changes in the flexibility of the probe in I(12,3)-labeled intestinal cell membranes but also to define low probe/membrane protein ratios to ensure that probe-probe interaction effects would not interfere with the determination of the membrane fluidity and polarity.

Membrane fluidity comparisons. Spin-label experiments which involved a comparison of the fluidity of two or more I(12,3)-labeled intestinal membranes were conducted on the same day to eliminate any error due to day-to-day fluctuations in sample temperature. Duplicate spectra were recorded at 37°C from each membrane suspension labeled with an experimentally-determined low probe/membrane protein ratio. Low probe-membrane protein ratio ranges were determined by titrating each examined membrane preparation with I(12,3) [35]. If low probe/membrane protein ratios could not be obtained unless the total probe concentration in the sample cavity was diluted to a level that noticeably decreased the signal/noise ratio, then the membrane was instead increased in concentration and again titrated with probe. This procedure ensures accurate determinations of low probe/membrane protein ratio ranges at suitable signal/noise levels.

Assays. Protein was determined according to the procedure of Lowry et al. [36], with the exception that all samples were made 0.4% in SDS prior to the addition of the alkaline copper tartrate solution.

Sucrose (EC 3.2.1.48) activity was measured according to a modification of the procedure of Dahlquist [37]. Duplicate samples were prepared in a total volume of 0.1 ml. To each sample was added 0.1 ml of substrate (0.056 M sucrose in 0.1 M malate, pH 6.0). One sample, the substrate blank was immediately immersed in a boiling water bath for 2 min; the other was incubated at 37°C for 45 min prior to immersion in boiling water. Precipitated protein was removed by centrifuga-

tion and the resulting supernatant fraction was assayed for liberated glucose by the addition of 4 ml of glucose oxidase-peroxidase reagent. (Sigma serum glucose determination kit No. 510 prepared in 0.5 M Tris-HCl, pH 7.0). Sucrase activity was defined as the difference in liberated glucose between the incubated sample and the substrate blank.

(Na^+, K^+)-ATPase activity was defined as the difference in total ATPase activity in the presence and absence of ouabain and was measured according to the procedure of Fujita et al. [38].

Alkaline phosphatase (EC 3.1.3.1) activity was measured as the rate of hydrolysis of *p*-nitrophenylphosphate. The final assay volume of 0.2 ml contained 21 mM glycine, pH 9.2, 2.1 mM $MgCl_2$, 0.42 mM $ZnCl_2$, 77.5 mM *p*-nitrophenylphosphate. The reaction was carried out at 37°C and terminated by the addition of 2.5 ml 0.02 M NaOH. The extent of hydrolysis was monitored at 400 nm and quantitated by using the molar extinction coefficient of *p*-nitrophenylate (18300). Succinate dehydrogenase (EC 1.3.99.1) was assayed according to the procedure of Pennington [39].

Galactosyl transferase (EC 2.4.1.38) activity was determined by a modification of the procedure of Kim et al. [40]. Alterations in this procedure included the absence of 2-mercaptoethanol in the incubation buffer and the use of ovalbumin as an artificial galactose acceptor. Transfer of [6-^{3}H]galactose from UDP-[6-^{3}H]galactose to ovalbumin was determined by trichloroacetic acid precipitation of the ovalbumin at the end of the incubation period.

Lipid extracts of brush border membranes were assayed for cholesterol and lipid phosphorus as described by Stadtman [41] and Bartlett [42], respectively.

Results

Membrane purification

Table I lists the specific activities of various marker enzymes in the homogenate and purified membrane fractions derived from vitamin D-replete and vitamin D-deficient chicks. Also reported is the fold increase or decrease in specific activities of marker enzymes in the purified membrane fractions relative to the homogenate. Sucrase and alkaline phosphatase are marker enzymes for brush border membranes while (Na^+, K^+)-ATPase is the marker enzyme for basal lateral membranes. Succinate dehydrogenase, galactosyl transferase and cytochrome *c* reductase are marker enzymes for mitochondria, Golgi and endoplasmic reticulum membranes, respectively. The effectiveness of the isolation procedure will be addressed in the Discussion.

Effects of essential fatty acid deficiency

The fatty acid composition analysis of lipids extracted from scraped mucosa is presented in Table II. As can be seen, essential fatty acid deficiency results in a significant alteration of the fatty acid patterns of the mucosal lipids. In the essential fatty acid-deficient group the levels of linoleic acid were reduced to 50% of the essential fatty acid-containing groups. Arachidonic acid levels remained low, while myristic, palmitoleic and eicosatrienoic acid appeared in measurable amounts in the essential fatty acid-deficient groups, but were absent in the essential fatty acid-containing groups. Table II also demonstrates that essential fatty acid deficiency did not differentially alter the fatty acid composition of vitamin D-replete versus vitamin D-deficient groups.

To properly evaluate the effect of essential fatty acid deficiency on intestinal calcium transport, it is necessary to first determine if essential fatty acid deficiency interferes with the metabolism of vitamin D. Fig. 1 shows the production in vitro of $1,25(OH)_2D_3$ by kidney homogenates from the four experimental groups. Both essential fatty acid-deficient and essential fatty acid-containing groups were able to convert 25-hydroxyvitamin D-3 to $1,25(OH)_2D_3$. The rates of production of $1,25(OH)_2D_3$ were lower in those homogenates which were derived from chicks dosed with vitamin D-3 as has been reported in detail by Henry et al. [43].

In Table III is shown the effect of essential fatty acid deficiency on three vitamin D-modulated calcium parameters. Essential fatty acid deficiencies did not affect the increase in serum calcium in response to vitamin D. The vitamin D replete-to-deficient ratio of serum calcium in the essential fatty acid-deficient groups is 1.6. This is essentially identical to the ratio of 1.7 found in the essential

TABLE I

SPECIFIC ACTIVITIES OF MEMBRANE MARKER ENZYMES IN THE HOMOGENATE AND PURIFIED MEMBRANE FRACTIONS OF INTESTINAL EPITHELIAL CELLS DERIVED FROM VITAMIN D-REPLETE (+D) AND DEFICIENT (−D) CHICKS

H, homogenate; BL, basal lateral membranes; BB, brush border membranes. All activities are expressed as nmol of product produced/min per mg protein+S.E. at 37°C. Galactosyl transferase activity is expressed as pmol [6-^{3}H]galactose transferred/h per mg protein. The values in parenthesis indicate the number of determinations. The values in brackets indicate the purification of the appropriate marker enzyme in the purified membranes relative to the homogenate. n.d. means not detectable.

		Sucrase	(Na^+, K^+)-ATPase	Alkaline phosphatase	Maltase	Succinate dehydrogenase	Galactosyl transferase
H	−D	42 ± 2 (18)	95 ±10 (3)	360± 47 (6)	55± 12 (3)	74 ±9 (3)	4.9±0.5 (3)
	+D	24 ± 3 (7)	90 ±17 (3)	1800± 370 (3)	33± 4 (3)	82 ±3 (3)	5.4±1.7 (3)
BL	−D	16.1± 3.3 (7) [0.38]	480 ±15 (3) [5.07]	400± 12 (3) [1.1]	96± 14 (3) [1.75]	23 ±4 (3) [0.31]	32 ±7 (3) [6.5]
	+D	7.7± 1.8 (3) [0.32]	390 ±33 (3) [4.38]	1030± 98 (3) [0.58]	73± 13 (3) [2.2]	35 ±5 (3) [0.41]	14 ±4 (3) [2.6]
BB	−D	550 ±42 (15) [13.1]	2.6± 0.2 (3) [0.03]	4800± 260 (3) [13]	3360±359 (3) [65]	1.4±0.5 (3) [0.02]	n.d. (4)
	+D	200 ±20 (7) [8.3]	2.5± 0.3 (3) [0.03]	21000±7000 (3) [12]	1800± 25 (3) [55]	4.0±1.1 (3) [0.05]	n.d. (3)

TABLE II

EFFECT OF ESSENTIAL FATTY ACID DEFICIENCY ON THE FATTY ACID COMPOSITION OF MUCOSAL LIPIDS FROM VITAMIN D-REPLETE OR VITAMIN D-DEFICIENT CHICKS

Fatty acid methyl esters of mucosal lipids were prepared and analyzed by GLC as described in Materials and Methods. The fatty acid composition was quantitatively determined by measuring the areas of the peaks with the aid of a planimeter. Chicks were raised on vitamin D-deficient (−D) diets which were essential fatty acid-replete (+EFA) or essential fatty acid-deficient (−EFA). Groups designated +D received 500 I.U. (32.5 nmol) of vitamin D-3 65, 98 and 24 h prior to death. Pooled samples from five to six chicks were used in each group. Similar results were obtained in two additional experiments.

Group	Fatty acids (% of total lipids)							
	Myristic 14:0	Palmitic 16:0	Palmitoleic 16:1	Stearic 18:0	Oleic 18:1	Linoleic 18:2	Eicosa-trienoic 20:3	Arachidonic 20:4
+D, +EFA	-	16.5	-	22.4	15.0	41.9	-	4.1
+D, −EFA	3.9	21.5	2.4	24.5	23.2	20.0	1.0	3.4
−D, +EFA	-	20.1	-	22.2	14.5	39.0	-	3.7
−D, −EFA	2.7	21.4	2.3	27.1	26.9	15.6	1.0	3.7

fatty acid-containing groups. Similar to serum calcium, the increase in calcium transport in vivo in response to vitamin D is not altered by essential fatty acid deficiency. Vitamin D administration enhances calcium absorption 4–5-fold in both essential fatty acid-containing and essential fatty acid-deficient groups. The vitamin D-replete/vitamin D-deficient ratio of $^{45}Ca^{2+}$ in a 0.2 ml aliquot of serum is 4.7 in the essential fatty acid-containing group versus 4.0 for the essential fatty acid-deficient group.

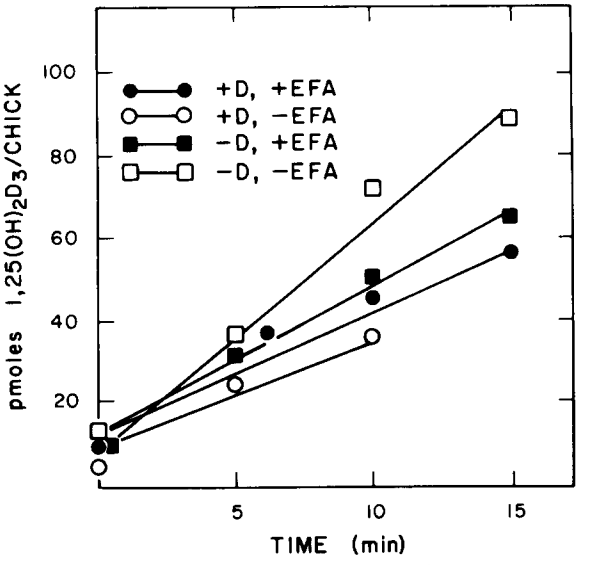


Fig. 1. Production in vitro of $1,25(OH)_2D_3$. Aliquots of a 10% homogenate of kidney from three chicks were incubated with 1000 pmol of 25-hydroxy[26,27-^{3}H]vitamin D-3. At the indicated time points an aliquot of the incubation mixture was removed and the conversion of 25-hydroxyvitamin D-3 to $1,25(OH)_2D_3$ was determined. Chicks were fed a vitamin D-deficient diet (−D) which was made essential fatty acid-replete or -deficient (+EFA or −EFA). Vitamin D-replete chicks (+D) received 500 I.U. (32.5 nmol) of vitamin D-3 72, 48 and 24 h prior to death.

In contrast to both serum calcium and calcium transport in vivo, the vitamin D-dependent modulation of calcium flux as measured in vitro is dependent on the essential fatty acid status of the chick. Calcium flux in the essential fatty acid-containing groups show a significant increase ($P > 0.01$) in response to vitamin D administration with a vitamin D-replete/vitamin D-deficient ratio of 1.8. However, vitamin D administration to chicks in the essential fatty acid-deficient groups results in no significant increase in the calcium flux as measured under in vitro conditions. The vitamin D-replete/vitamin D-deficient ratio in the essential fatty acid-deficient group is 1.0.

Effect of vitamin D on brush border membrane fluidity

Similar to previous results observed with I(12,3)-labeled membranes [30,31,44,45], probe-probe interaction effects occur when intestinal membrane fractions were labeled with probe concentrations above the low probe/membrane protein ratio

TABLE III

EFFECT OF ESSENTIAL FATTY ACID DEFICIENCY ON SERUM CALCIUM, CALCIUM TRANSPORT IN VIVO AND CALCIUM FLUX IN VITRO

For description of the groups see Table II. The values are expressed as an average ± S.D. The number of determinations is given in parenthesis.

Group	Serum calcium		Calcium transport		Calcium flux in vitro	
	mg/100	+D/−D	$^{45}Ca^{2+}$/0.2 ml of serum (dpm)	+D/−D	nmol Ca^{2+}/h per segment	+D/−D
+D, +EFA	9.9 ± 1.6 (10) *	1.7	3390 ± 380 (5) *	4.7	0.80 ± 0.34 (7) **	2.8
−D, +EFA	5.7 ± 1.4 (12)		720 ± 220 (5)		0.19 ± 0.08 (7)	
+D, −EFA	8.8 ± 1.8 (10) *	1.6	3600 ± 700 (5) *	4.0	0.73 ± 0.24 (7)	1.02e
−D, −EFA	5.5 ± 1.6 (11)		890 ± 340 (5)		0.75 ± 0.75 (7)	

* Significantly different from the value observed in the corresponding −D group ($P > 0.001$).
** Significantly different from corresponding −D group ($P > 0.01$).

range. The results of experiments designed to determine an optimum probe/membrane protein ratio for intestinal brush border membranes are shown in Fig. 2. S and $S(T_\perp)$ clearly decrease substantially if the I(12,3) concentration exceeded 15 nmol probe per 700 μg membrane protein. Moreover, $S(T_\parallel)$ values were relatively independent of the probe concentrations employed in the membrane over a wide range of probe/membrane protein ratios. Qualitatively similar results were obtained for the basal lateral and 'Golgi' membrane fractions, where S and $S(T_\perp)$ diverged from $S(T_\parallel)$ at probe/membrane ratios exceeding 10 and 15 nmol probe/297 μg membrane protein, respectively. The probe concentration-dependent decreases in S and $S(T_\perp)$ are due to probe-probe interaction effects on the ESR spectra and are not the result of probe mediated perturbations in the membrane structure [31,35].

Fig. 3 indicates a representative membrane fluidity comparison study in which order parameters of the labeled intestinal cell membrane fractions, prepared from vitamin D-deficient and replete chicks, were calculated at 37°C. The brush border membrane order parameters S, $S(T_\parallel)$, and $S(T_\perp)$ are much greater than the corresponding order parameters obtained for the basal lateral and Golgi membrane preparations. The difference in the order parameter S between brush border and basal lateral (or Golgi) membranes is in the order of $\Delta S = 11\%$; we have found for a wide variety of I(12,3)-labeled biological membranes [11,31,35] that a decrease in membrane temperature from 37°C to approx. 26°C would be required to increase S by such an amount. Identical findings were obtained whether freshly-prepared mem-

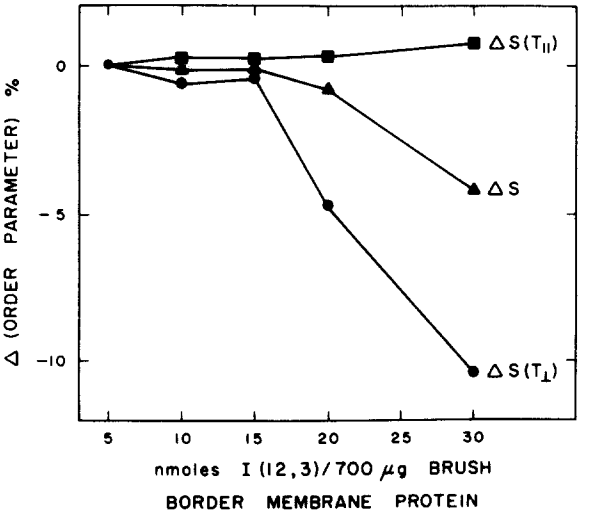


Fig. 2. Effects of probe concentration on the order parameters of I(12,3)-labeled chick intestinal epithelial cell brush border membranes at 37°C. The percent changes in the order parameters from baseline values measured at 5 nmol I(12,3)/700 μg membrane protein, are plotted as a function of the probe/membrane protein ratio. The baseline values of $S(T_\parallel)$, $S(T_\perp)$, and S were 0.710, 0.600 and 0.640, respectively. The order parameters were measured from the spectra of the I(12,3)-labeled membranes as described in Materials and Methods.

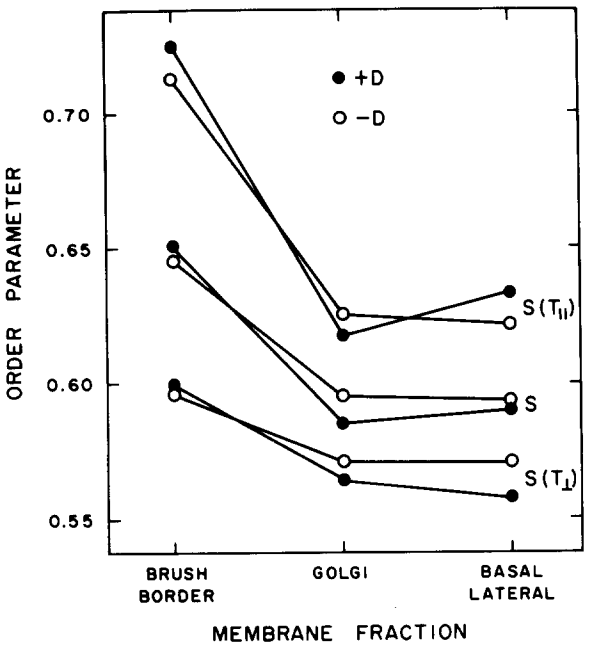


Fig. 3. Effect of vitamin D status on order parameter values from I(12,3)-labeled brush border, Golgi and basal lateral membranes. S, $S(T_{\parallel})$ and $S(T_{\perp})$ were calculated from duplicate spectra recorded at 37°C as described in Materials and Methods. Vitamin D-replete chicks (+D) received 500 I.U. of vitamin D-3 96, 72, 48 and 24 h prior to death. Vitamin D-deficient chicks (−D) received the solvent vehicle.

branes or membranes stored frozen at −70°C were examined. It is evident from the figure that the order parameters of the three membranes were unaffected by changes in vitamin D status of the chick.

The isotropic hyperfine coupling constants were tabulated for the brush border, basal lateral and Golgi membrane fractions prepared from vitamin D-deficient and vitamin D-replete chicks. The a'_N values are listed in Table IV. It is apparent that the polarity of the environment of the probe in the brush border membrane is much greater than that of the basal lateral or Golgi preparations. This brush border/basal lateral difference is apparent in both the vitamin D-deficient and replete animals. However, the a'_N values for each given membrane were in quite close agreement between the vitamin D-replete and vitamin D-deficient states; the isotropic hyperfine coupling constants obtained from a study in which the vitamin D-deficient and replete chick membranes were directly compared are shown for the brush border membrane.

TABLE IV

INTRINSIC ISOTROPIC HYPERFINE COUPLING CONSTANT (a'_N) VALUES DETERMINED AT 37°C FROM CHICK INTESTINAL MEMBRANE FRACTIONS

The values represent the mean±1 S.D. for data from several membrane preparations obtained from vitamin D-deficient (−D) or vitamin D-replete (+D) birds labeled with low (see Materials and Methods) probe/membrane protein ratios. The number of values averaged is indicated in parentheses. Results from a representative comparison study of vitamin D-deficient and replete chick brush border membrane are included.

Membrane fraction	a'_N
Brush border	15.608±0.070 (11)
−D	15.542
+D	15.567
Basal lateral	15.209±0.066 (6)
Golgi fraction	15.193±0.034 (7)

Earlier studies suggest a relationship between the membrane fluidity and cholesterol content (or cholesterol/phospholipid ratio) of the membrane [44,46,47]. We therefore determined the cholesterol and phospholipid content of the brush border membranes used for spin label analysis. This data is shown in Table V. It is apparent that there is no significant difference in either of these membrane

TABLE V

EFFECT OF DIETARY VITAMIN D-3 ON THE BRUSH BORDER MEMBRANE CONTENT OF CHOLESTEROL AND LIPID PHOSPHORUS

Numbers represent the average±S.D. of three separate determinations. Brush border membrane fractions were isolated (see methods) from the duodenum of vitamin D-deficient (−D) or vitamin D-deficient chicks which received 500 I.U. (32.5 nmol) of vitamin D-3 96, 72, 48 and 24 h before death (+D).

Vitamin status	Cholesterol (μmol/mg protein)	Lipid phosphorus (μmol/mg protein)	Cholesterol/ lipid phosphorus
+D	0.27±0.03	0.19±0.01	1.4
−D	0.26±0.01	0.19±0.02	1.4

TABLE VI

ORDER PARAMETERS OBTAINED FROM VARIOUS I(12,3)-LABELED BIOLOGICAL MEMBRANE SYSTEMS AT 37°C

The order parameters were determined from membrane samples suspended in Tris-HCl buffer at physiological pH. Rat heart plasma membranes and adipocyte ghosts were prepared and labeled as described previously [30,31]. Human platelet plasma membranes, purified according to Barber and Jamieson [48] and human erythrocyte ghosts, prepared as described by Dodge et al. [49], were labeled as indicated earlier [31]. Rat liver plasma membrane preparations were purified according to Pilkis et al. [50] by Houslay and co-workers [11,51]. The chick intestinal cell brush border and basal lateral membranes were prepared as described in Materials and Methods. S, $S(T_{\parallel})$, and $S(T_{\perp})$ were calculated as indicated in text.

Membrane system	$S(T_{\parallel})$	S	$S(T_{\perp})$	Cholesterol/ phospholipid
1. Egg yolk lecithin dispersion	0.554	0.561	0.567	0
2. Chick intestinal cell basal lateral membrane	0.612	0.583	0.562	-
3. Rat heart plasma membrane	0.618	0.587	0.563	0.32
4. Rat epididymal fat cell ghosts	0.619	0.589	0.560	-
5. Rat liver plasma membranes	0.641	0.591	0.552	0.65
6. Human platelet plasma membranes	0.644	0.604	0.574	0.47
7. Human erythrocyte ghosts	0.691	0.638	0.600	0.81
8. Chick intestinal cell brush border	0.720	0.650	0.595	1.4

parameters as a function of vitamin D status of the animal. This correlates with the absence of any change in the order parameters of the I(12,3)-labeled membranes in response to vitamin D.

The cholesterol/phospholipid ratios and average order parameter S, $S(T_{\parallel})$ and $S(T_{\perp})$ values determined at 37°C are listed in Table VI for the intestinal cell brush border membrane, together with results from a wide variety of other biological membranes that we have examined with the spin label method (see Discussion).

Discussion

The results presented in this paper demonstrate that essential fatty acid-deficiency in the chick results in an incorporation of the short chain fatty acid, myristic acid and the unsaturated fatty acids, palmitoleic and eicosatrienoic acid in compensation for the loss of linoleic acid (see Table II). This substitution might reflect a compensation mechanism, similar to that of the unsaturated fatty acid auxotroph of *Escherichia coli* [50], which enables the intestinal mucosal membranes to maintain their physical and transport properties in vivo despite an alteration in the fatty acid composition. This concept is supported by the data in Table II which shows that essential fatty acid deficiency does not affect the ability of the chick to respond to vitamin D with an increase in intestinal calcium transport in vivo. In addition essential fatty acid deficiency does not prevent the vitamin D-dependent increase in serum calcium.

This data would appear to be in direct conflict to a recent report by Hay et al. [7] which argues that the vitamin D-mediated increase in intestinal calcium transport is essential fatty acid-dependent. However, the data reported by Hay et al. were obtained by measuring calcium fluxes in vitro. Indeed, when we measured calcium flux in vitro there was an apparent essential fatty acid-dependency to the vitamin D-dependent increase (see Table III). It is our opinion that in vivo calcium transport and serum calcium are the more physiological calcium homeostatic parameters. The apparent differential effect of essential fatty acid deficiency on calcium transport in vitro in ileum from vitamin D-replete versus vitamin D-deficient chicks may result from an instability of the brush border membrane surface in the absence of essential fatty acids. A number of studies offer evidence that the membrane stability of essential fatty acid-deficient membranes is affected by stress in vitro [53–55]. Also this laboratory's previously reported effects of filipin-stimulated intestinal calcium transport [17,18] could only be obtained by in

vitro application of the polyene antibiotic; no effects of filipin on $^{45}Ca^{2+}$ absorption could be noted when the antibiotic was placed in ligated loops in vivo.

Spin label studies on the intestinal epithelial cell membranes require that these membranes be isolated. Procedures for the preparation of mammalian brush border membranes [28,56–58] were found to be unsatisfactory when applied to the chick. It was therefore necessary to develop a new procedure for the isolation of chick brush border membranes. The procedure described here gives brush border membranes in a 25–30% yield. Membranes derived from control vitamin D-deficient chicks have a 13-fold increase in sucrase specific activity as compared to the starting homogenate. The sucrase specific activity in control brush border membranes is 550; this is very comparable to the value of 560 reported by Max et al. [29]. These membranes have very little contamination by succinate dehydrogenase and (Na^+, K^+)-ATPase. Galactosyl transferase activity was not detectable in purified brush border membranes. Alkaline phosphatase and maltase, which are localized in the brush border membrane, are co-purified with sucrase. Brush border membranes derived from vitamin-D-replete chicks show similar increases and decreases in marker enzyme specific activities as compared to membranes derived from vitamin D-deficient chicks. Alkaline phosphatase shows a marked increase in specific activity in response to vitamin D which is consistent with the vitamin D-dependent nature of this enzyme [59]. Brush border sucrase specific activity has been previously reported to be decreased in response to 1α-hydroxyvitamin D-3 treatment [29]. Table I verifies that this effect is also observed and is, in fact, more pronounced in response to vitamin D-3. This decrease is observed in the homogenate as well as the brush border membranes. Maltase also exhibits a similar vitamin D-dependent decrease in activity.

Electrophoretic analysis of membrane protein composition in the brush border and basal lateral membranes is shown in Fig. 4. The distinctly different protein migration patterns for these two membrane fractions clearly reflects a divergent biochemical differentiation which is presumably related to their different cellular localization and

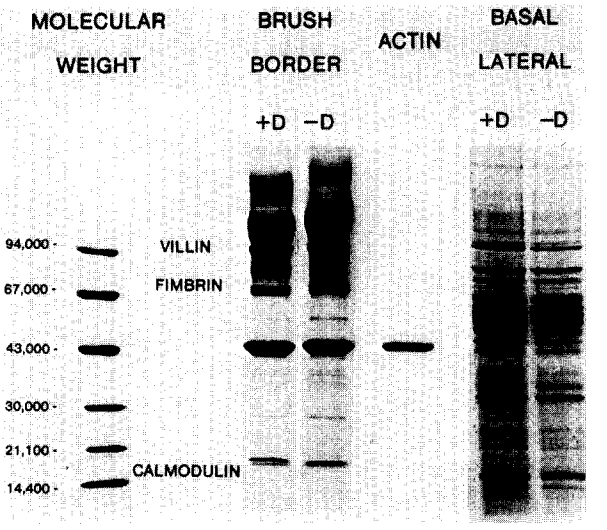


Fig. 4. SDS slab gel analysis of brush border and basal lateral membranes. Membranes were solubilized as described by O'Farrell [60] and separated on a 7–20% exponential gradient SDS slab gel. Electrophoresis buffers were prepared as described by Laemmli [61]. The gels were run with constant power at an initial current of 25 mA and subsequently stained with Coomassie Blue R-250.

functions. Brush border membranes derived from vitamin D-replete and vitamin D-deficient chicks exhibited modest changes in apparent composition of membrane proteins with molecular weights of 83000 and 77000 *.

The fluidity of the I(12,3)-labeled intestinal cell brush border and basal lateral membranes may be compared with the corresponding fluidities of other, similarly-labeled biological membranes. Table VI indicates intrinsic S, $S(T_{\parallel})$ and $S(T_{\perp})$ values for chick intestinal cell brush border and basal lateral membranes, rat liver and heart plasma membranes and adipocyte 'ghosts', and human platelet and erythrocyte ghost plasma membranes. The membranes were labeled at 37°C with low I(12,3) concentrations that were experimentally determined by titrating each membrane with spin probe (see Materials and Methods). The respective polarity-corrected order parameter S values, and the order parameters $S(T_{\parallel})$ and $S(T_{\perp})$, each indicated that the chick intestinal cell brush border membrane is much less fluid than the erythrocyte

* Putkey, J.A. and Norman, A.W., manuscript in preparation.

ghosts. Moreover, the basal lateral membrane is much more fluid than the rat liver plasma membrane and human platelet plasma membrane lipid sampled by the I(12,3) probe.

The chick brush border membrane is not only the most rigid membrane we have examined, but it also contains the highest cholesterol/phospholipid (C/PL) ratio. The order parameter S was thus plotted against the C/PL ratio, as shown in Fig. 5, to determine if this might provide a structural basis for the membrane fluidity differences observed in Table VI. In an earlier study, a similar analysis was made of the order parameter $S(T_{\|})$ values and the C/PL ratios for I(12,3)-labeled membranes at 30°C [44]. All of the available data suggest that the C/PL ratio may be involved in

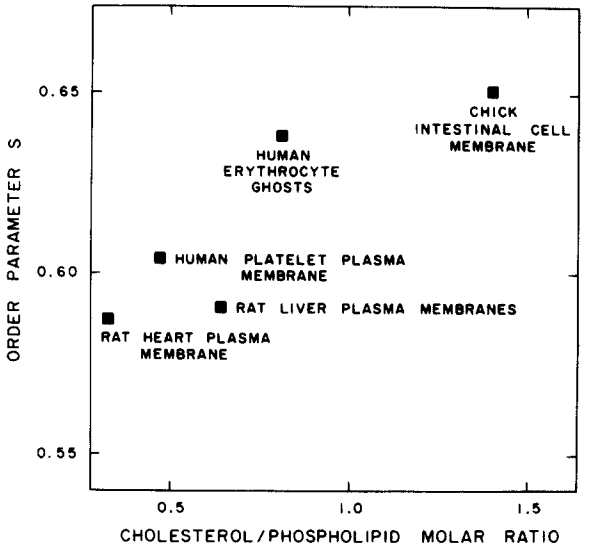


Fig. 5. An S vs. cholesterol/phospholipid (C/PL) molar ratio plot for several I(12,3)-labeled biological membranes at 37°C. The C/PL ratios for erythrocytes and platelet plasma membranes were obtained from Dodge and Phillips [62] and Barber and Jamieson [48], respectively, as described earlier [44]. The C/PL ratio for rat heart plasma membranes was determined by Feldman and Weinhold [63]. A wide scatter in the reported C/PL ratios for liver plasma membranes has been reported (0.26 to 0.70) [64] possibly due to differences in the purity of the preparations; the liver plasma membrane C/PL ratio reported here is an average value determined from several batches of liver plasma membrane prepared according to Pilkis at al. [50], Marchmont and Houslay [51], Houslay et al. [64], as were the membranes prepared for spin label studies. The polarity-corrected order parameter S was calculated, as indicated in Table V and the text, from membranes labeled with 'magnetically-dilute' probe concentrations.

the fluidity differences in Table VI since S increases with the C/PL ratio.

Jain and White [9] recently reviewed evidence that the lipids of cellular biological membranes may be viewed structurally as forming an 'ordered' fluid. In numerous instances, discrete ordering of lipid molecules into domains within a relatively fluid matrix have been detected even at temperatures above 'classical' order-disorder transition temperatures. Our results suggest that the lipids of the chick intestinal epithelial cell surface membrane are arranged into regions having widely differing fluidities. This is emphasized in Fig. 3 and Table VI which show the striking lipid fluidity differential between the brush border and basal lateral membranes. This difference in fluidity is not related to differing degrees of purity between these membrane fractions.

Brasitus and Schachter [16] recently examined by fluorescence polarization the lipid structures of rat enterocyte microvillus and basal lateral membranes labeled with several exogenous fluorescent lipid probes. Five fluorescent labels of differing structure were employed and in each case lipid molecules in the basal lateral membrane exhibit greater motional freedom than the brush border membrane. Our findings with I(12,3)-labeled chick intestinal cell membranes suggest that spin and fluorescent labels in these studies report qualitatively similar information. Moreover, the dynamic lipid properties reported by the labels are likely related to significant structural features of these membranes and are not artifacts due to the incorporation of the perturbing labels in the membrane.

It must be emphasized that the differences in the lipid dynamic structures of the brush border and basal lateral plasma membranes may not necessarily be related solely to the different cholesterol/phospholipid (C/PL) ratios found for these membranes. Brasitus and Schachter [16] pointed out for the rat intestinal enterocyte membranes that such composition changes as the sphingomyelin/phosphatidylcholine and the protein/lipid ratios of the membranes might be involved in the fluidity differences observed in this system. These and other structural parameters, such as the ratio of unsaturated/saturated fatty acids in the membrane and the membrane content of divalent cations such as Ca^{2+} and Mg^{2+}, have

previously been correlated with alterations in the lipid fluidity [30,35,44,65–67].

The polyene antibiotic filipin increases the in vitro transepithelial flux of calcium in intestine obtained from vitamin D-deficient chicks [17,18]; and *cis*-vaccenic acid increases calcium uptake into brush border membrane vesicles isolated from vitamin D-deficient chicks [20]. Both these amphipathic agents have a fluidizing effect on membrane lipids [19]. It could therefore be rationalized that vitamin D effects an increase in intestinal calcium transport by increasing the fluidity of brush border membrane lipids. Yet, we did not observe a vitamin D-dependent alteration in the lipid fluidity of the brush border, basal lateral or Golgi membranes (see Fig. 3) when studied by ESR using I(12,3) as a spin label. There are, however, experimental limitations which could obscure a change in the lipid fluidity. The spin label probe I(12,3) may not be able to detect minor vitamin D-dependent alterations in membrane lipid fluidity. Alternatively, I(12,3) may not partition into the subdomain of lipid which is modified by vitamin D. The recent results of Klausner et al. [68] emphasize the importance of lipid subdomains in affecting the fluidity and properties of biological membranes. This particular problem could be surmounted with the use of a variety of spin label probes.

In summary, the data presented in this report do not support a role for essential fatty acids in mediating the in vivo increase in intestinal calcium transport in response to vitamin D. The application of ESR spectroscopy to the study of intestinal epithelial cell surface membranes confirms the distinct lipid structural differences between the brush border and basal lateral membranes which undoubtedly play a prominent role in maintaining the functional differences between these two membranes. In addition, the brush border membrane has a uniquely high lipid rigidity and a correspondingly high cholesterol/phospholipid molar ratio. In light of a possible lipid mediated regulation of intestinal calcium transport we felt the need existed for the utilization of techniques, such as ESR, to directly detect putative vitamin D-dependent alterations in physical state of membrane lipids. With the use of the spin label probe I(12,3) we were unable to detect a change in the lipid fluidity of the intestinal epithelial cell membrane which suggests that the vitamin D-dependent increase in intestinal calcium transport is mediated via mechanisms which do not involve a change in this membrane parameter. Additional studies utilizing spin label probes capable of sampling a variety of lipid domains will be necessary to confirm this conclusion.

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