Relationship of non-esterified fatty acids to vitamin D-dependent Ca^{2+} binding by rat intestinal Golgi-enriched membrane fractions

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Ca^{2+} binding and concentrations of non-esterified fatty acids and phospholipids were compared in membrane fractions of rat small intestine. These fractions differed in density and were enriched for galactosyltransferase activity, a Golgi-membrane marker. Ca^{2+} binding was highest in the Golgi subfraction with the least density, as were the concentrations of both non-esterified fatty acids and phospholipids; galactosyltransferase activity was distributed differently. The large amount of non-esterified fatty acids was sufficient to account for a 2:1 complex of fatty acid– Ca^{2+} . In vitamin D-deficient animals, the yield of protein in the lightest subfractions was decreased, but Ca^{2+} binding per mg of protein was further decreased to about 60%. In Golgi fractions from vitamin D-deficient animals, Ca^{2+} binding and the concentration of non-esterified fatty acids were decreased in parallel, but phospholipids were not significantly changed. There was a close correlation between Golgi Ca^{2+} binding and non-esterified fatty acid concentrations ($r = 0.89$; $P < 0.001$). Non-esterified fatty acids, which are unusually prevalent in these membrane fractions, are likely to be the binding sites that account for this vitamin D-dependent Ca^{2+} uptake.

In the preceding paper, the characteristics of vitamin D-dependent Ca^{2+} uptake by intestinal Golgi-enriched membrane fractions were shown to be incompatible with binding to proteins, and it was suggested that the Ca^{2+} uptake could represent binding to lipids, such as phospholipids or non-esterified fatty acids (Walters & Weiser, 1984). Phospholipid binding of Ca^{2+} has been extensively studied in other biological systems (see, e.g., Seimiya & Ohki, 1973). By comparison with those studies, however, the characteristics of the binding seen in these Golgi fractions, particularly the pK_a of the binding site, suggested that the binding site might consist not of phospholipids but rather of non-esterified fatty acids. Consequently, we studied the relationship between non-esterified fatty acids, total phospholipids and Ca^{2+} uptake in (i) subcellular membrane fractions prepared from normal animals and (ii) combined Golgi fractions prepared from normal and vitamin D-deficient animals. These studies indicate that the predominant Ca^{2+} -binding sites in these membrane fractions are non-esterified fatty acids.

Experimental

Membrane preparation

Male weanling rats were fed on a vitamin D-deficient or a control vitamin D-sufficient diet until 5–8 weeks of age, and intestinal membrane fractions were then prepared from scrapings of entire small intestine as described previously (Walters & Weiser, 1984). Discontinuous-sorbitol-density-gradient centrifugation was used to separate four fractions. Three of these, the sorbitol 20g/dl fraction (density ≤ 1.06 g/ml), the 20g/dl–30g/dl interface (density between 1.06 and 1.10 g/ml) and the 30g/dl fraction (density = 1.10g/ml) were those that had been combined as the Golgi fraction in previous studies. In some experiments reported here, they were also collected as a single, combined fraction. The other subfraction, the 30g/dl–40g/dl interface (density between 1.10 and 1.13g/ml), although slightly enriched with galactosyltransferase activity, contained significantly more ($\text{Na}^+ + \text{K}^+$)-dependent ATPase activity, a marker for baso-lateral membranes (Freedman *et al.*, 1977; Weiser *et al.*, 1978). The membrane

fractions were washed and separate portions were used for determinations of Ca^{2+} uptake, galactosyltransferase activity and protein and lipid concentrations.

Ca^{2+} uptake was measured with $^{45}\text{Ca}^{2+}$ and nitrocellulose filtration as described by Walters & Weiser (1984). All uptake values were determined after incubation for 30 min with 0.4 mM total calcium; this uptake value is similar to the amount of Ca^{2+} bound to the high-affinity sites at equilibrium, but underestimates total binding by a factor of 1.7 ± 0.1 (mean $\pm$ S.E.M.). Protein was measured by the method of Bradford (1976), and galactosyltransferase activity as described by Podolsky & Weiser (1975).

Lipid extraction and measurement

Portions of membrane fractions, frozen immediately after preparation, were thawed and their lipids were extracted with methanol/chloroform by the method of Bligh & Dyer (1959). Extracts equivalent to 100 μg of protein were separated by t.l.c. with a solvent system of light petroleum (b.p. 35–60°C)/diethyl ether/acetic acid (90:10:1, by vol.) as described by Skipski & Barclay (1969). Non-esterified fatty acids and triacylglycerols were identified after brief I_2 exposure by comparison with simultaneously run oleic acid and trioleoylglycerol standards (Supelco, Bellefonte, PA, U.S.A.). Clear separation occurred between these and the adjacent spots. The fatty acid and triacylglycerol spots were scraped off the chromatogram and quantified by dichromate oxidation (Skipski & Barclay, 1969), by using 0.5 ml of reagent and the oleic acid and trioleoylglycerol standards. Phospholipids were measured as total lipid phosphate by the method of Bartlett (1959), by using the spot that remained at the origin of the chromatogram. Lipid separations and determinations were performed in duplicate.

Statistical analysis

Paired *t* tests were used to compare different fractions obtained from the same animal, and unpaired *t* tests to compare the two dietary groups. Results are expressed as means $\pm$ S.E.M. Linear regression analysis was by standard non-weighted methods.

Results

Subfractions in normal animals

The distribution in the four subfractions of Ca^{2+} binding and galactosyltransferase activity is shown in Fig. 1(a). Ca^{2+} binding was greatest in the least dense subfraction (20 g/dl) and was significantly less in each successively denser subfraction. Galactosyltransferase activity was differently dis-

Fig. 1. Membrane subfractions in normal animals (a) Distribution of Ca^{2+} binding and galactosyltransferase activity. (b) Concentrations of non-esterified fatty acids and total phospholipids. The four subfractions were separated by discontinuous-sorbitol-density-gradient centrifugation and the measurements made as described in the text. Results are means $\pm$ S.E.M. for six normal animals.

tributed; equal activities were found in the two least dense subfractions (20 g/dl and 20 g/dl–30 g/dl), and significantly decreased activities in each of the next two subfractions. The four subfractions were shown in a separate series of animals to be enriched for galactosyltransferase activity by factors of 9.6, 9.0, 4.1 and 2.7 when compared with homogenate (means for 11 animals).

Total phospholipids and non-esterified fatty acids were both distributed similarly to the Ca^{2+} binding in the various fractions (Fig. 1b). The greatest amount (relative to protein) was found in

Fig. 2. Correlation between non-esterified fatty acids and Ca^{2+} binding in the four membrane subfractions. The results from six normal animals for the four fractions are shown. Methods of measurement and the regression analysis are described in the text. The sorbitol-density-gradient fractions were as follows (g/dl): ●, 20; ○, 20/30; ■, 30; □, 30/40.

the least dense fraction and significantly less in each successive fraction with greater density. Over these four subfractions, there were significant correlations ($P < 0.001$) between individual measurements made in a group of six normal animals for non-esterified fatty acids, phospholipids and Ca^{2+} binding; $r = 0.82$ for non-esterified fatty acids and phospholipids; $r = 0.84$ for phospholipids and Ca^{2+} binding; and $r = 0.86$ for non-esterified fatty acids and Ca^{2+} binding (Fig. 2). The regression line for all the data points shown in Fig. 2 had a slope of 2.06. If the data showing non-esterified fatty acid values greater than $1.6 \mu\text{mol/mg}$ of protein were excluded (since the fatty acid assay, as performed, was not linear above this value) there was an increase in the slope to 3.16. Whichever value is used, however, there were more than enough non-esterified fatty acids present in these fractions to give a 2:1 complex of fatty acids to calcium.

Much of the correlation between non-esterified fatty acids, phospholipids and Ca^{2+} binding was due to the effect of the different mean compositions of the four subfractions. When an analysis of variance was performed and the effects of the different mean compositions of the four subfractions were removed, the residual variance still showed a positive correlation within the groups for non-esterified fatty acids and Ca^{2+} binding ($r = 0.49$, $P < 0.05$). No similar correlation was

Fig. 3. Protein yields for the four subfractions prepared from normal or vitamin D-deficient animals.

The mean $\pm$ S.E.M. for total protein recovery in each of the four subfractions prepared by sorbitol-density-gradient centrifugation is illustrated for six normal animals (shaded columns) and six vitamin D-deficient animals (open columns).

then found between phospholipids and Ca^{2+} binding or between non-esterified fatty acids and phospholipids.

Triacylglycerols were present in the 20 g/dl subfraction ($232 \pm 39 \text{ nmol/mg}$ of protein) and in the 20 g/dl–30 g/dl subfraction ($82 \pm 27 \text{ nmol/mg}$ of protein), but were not detected in significant quantity in the other subfractions.

Changes in subfractions in vitamin D deficiency

The decrease in Ca^{2+} uptake seen previously with combined Golgi fractions prepared from vitamin D-deficient animals could result from a selective decrease in the recovery of the subfractions with the greatest Ca^{2+} binding per mg of protein. Fig. 3 shows that there was in fact a significant ($P < 0.01$) decrease in protein recovery in the 20 g/dl and 20 g/dl–30 g/dl interface subfractions prepared from vitamin D-deficient animals. There was no significant change detected in the other two subfractions.

This altered recovery, however, was not the sole explanation for the decrease in Golgi Ca^{2+} binding seen in vitamin D deficiency. As shown in Fig. 4, there was a significant decrease in Ca^{2+} binding per mg of protein in every subfraction prepared from vitamin D-deficient animals. In the three subfractions that comprised the Golgi, this was remarkably constant at 58–60%. The decrease in the 30 g/dl–40 g/dl interface subfraction (72%) was less marked. Because of the low yield of membranes obtained in vitamin D-deficient animals, non-esterified fatty acids and phospholipids were not measured in the separate subfractions,

Fig. 4. Ca^{2+} binding for the four subfractions prepared from normal or vitamin D-deficient animals

The mean $\pm$ S.E.M. for Ca^{2+} binding in each of the four subfractions prepared by sorbitol-density-gradient centrifugation is shown for six normal animals (shaded columns) and six vitamin D-deficient animals (open columns).

but only in the combined Golgi and 30 g/dl–40 g/dl interface fractions.

Lipid concentrations in combined Golgi fractions in vitamin D deficiency

Ca^{2+} binding and concentrations of non-esterified fatty acids and total phospholipids were measured in combined Golgi fractions, i.e. the combined 20 g/dl, 20 g/dl–30 g/dl interface and 30 g/dl subfractions, prepared from six vitamin D-deficient animals and compared with six normal animals (Fig. 5). The decrease (to 48%) in mean Ca^{2+} bound by Golgi prepared from vitamin D-deficient animals was clearly similar to the decrease in mean non-esterified fatty acids (to 47%). The ratio of the vitamin D-dependent change in non-esterified fatty acids to the vitamin D-dependent change in Ca^{2+} binding was 727/196, i.e. 3.7. When the Ca^{2+} binding was corrected by a factor of 1.7 to approximate to total Ca^{2+} binding at equilibrium, the fatty acid/ Ca^{2+} ratio then became 2.2, close to the value of 2 expected for Ca^{2+} –fatty acid complexes. There was no significant change in the mean total phospholipids in combined Golgi fractions prepared from vitamin D-deficient animals.

The correlation between these individual animals' non-esterified fatty acids and Ca^{2+} binding is shown in Fig. 6. This was highly significant

Fig. 5. Ca^{2+} binding, non-esterified fatty acids and total phospholipids in Golgi prepared from normal or vitamin D-deficient animals

The mean $\pm$ S.E.M. is shown for the combined Golgi fractions (i.e. the combined 20 g/dl, 20 g/dl–30 g/dl and 30 g/dl subfractions) for six normal animals (shaded columns) and six vitamin D-deficient animals (open columns).

Fig. 6. Correlation between non-esterified fatty acids and Ca^{2+} binding in combined Golgi fractions

Results from six normal animals (●) and six vitamin D-deficient animals (○) are shown. The equation of the regression line is $y = 3.4x + 62$.

($r = 0.89$, $P < 0.001$) and the regression coefficient (3.4) again approximated that expected for Ca^{2+} –fatty acid complexes.

The 30 g/dl–40 g/dl interface fraction was also studied. The decrease in non-esterified fatty acids did not reach statistical significance ($0.1 > P > 0.05$), but there was a significant increase ($P < 0.02$) in total phospholipids found in vitamin D-deficient animals, from 296 ± 43 to 469 ± 46 nmol/mg of protein. There was a negative correlation between Ca^{2+} binding and phospholipids in this fraction ($r = 0.73$, $P < 0.001$). When the values for this fraction were included with those for the combined Golgi fraction, the correlation between

non-esterified fatty acids and Ca^{2+} binding was little altered ($r = 0.85$, $P < 0.001$).

Discussion

The membrane subfractions studied in the present paper represented a simple modification of the methods previously used to prepare fractions enriched for the Golgi marker galactosyltransferase activity (Freedman *et al.*, 1977, 1981; Weiser *et al.*, 1978; MacLaughlin *et al.*, 1980; Walters & Weiser, 1984). The three separate subfractions that in the previous studies had been pooled were each shown here to take up Ca^{2+} , also in a vitamin D-dependent manner. The results with these subfractions could be combined to produce values similar to those obtained when the Golgi had not been divided.

The two subfractions with the least density, although similarly enriched for galactosyltransferase activity, differ in their ability to bind Ca^{2+} and also in their non-esterified fatty acid and phospholipid concentrations. This may indicate that different subsets of Golgi-derived membranes are involved and that the ability to bind Ca^{2+} is related to the lipid/protein ratio and thus to the density of the membrane subfraction. These two fractions with the largest amount of Ca^{2+} binding, non-esterified fatty acids and phospholipids are also the only ones to contain significant amounts of triacylglycerols. They may represent the portion of the Golgi involved in secretion of chylomicron particles (Green & Glickman, 1981; Miyamoto & Tanaka, 1982).

Total phospholipids in the subfractions were shown to correlate with Ca^{2+} binding, but this effect was lost when the different mean composition of each subfraction was taken into account. There was no significant positive correlation in different animals between Ca^{2+} binding and total phospholipids; indeed, in the 30 g/dl–40 g/dl interface fraction there is a significant negative correlation. The phospholipids that bind Ca^{2+} most strongly (phosphatidylserine, phosphatidylinositol and phosphatidic acid) comprise only a small proportion of total phospholipids in biological membranes, including those studied in the present paper (results not shown), and would not be sufficient to account for the magnitude of Ca^{2+} binding.

The association between non-esterified fatty acids and Ca^{2+} binding was appropriate in quantity; both in the various subfractions and in the different normal and vitamin D-deficient animals, there was more than sufficient non-esterified fatty acid present to form a 2:1 complex of fatty acid– Ca^{2+} . The Ca^{2+} bound to the high-affinity binding site [K_D approx. $3 \mu\text{M}$ (Walters &

Weiser, 1984)] is similar in number to Ca^{2+} uptake measured after 30 min at 0.4 mM total Ca^{2+} and is less than that which could complex with the total non-esterified fatty acids. Presumably not all of the fatty acids are available for this high-affinity binding, and the remainder of the binding seen at equilibrium is with lower affinity either to the residual fatty acids or to other sites. We have not further investigated this by localizing the non-esterified fatty acids to the inside or outside of the membrane fractions or to their contents, or by characterizing the types of fatty acids.

The amount of non-esterified fatty acids found in these Golgi fractions was surprisingly large [e.g. 0.34 mg (approx. $1.37 \mu\text{mol}$)/mg of protein in the combined fraction from normal animals]. This was unexpected, and we are uncertain of its relevance, or the way this is altered by the vitamin D status of the animal. Liver Golgi membranes, although they contain proportionally more non-esterified fatty acids than plasma membranes, do not have the high concentrations found in our preparation (Keenan & Morr, 1970). In guinea-pig pancreatic Golgi membranes a high concentration of non-esterified fatty acids was found, but was thought to be due to the action of pancreatic lipase on other membrane constituents during isolation (Meldolesi *et al.*, 1971). Intestinal Golgi lipid concentrations have not been determined previously. Douglas *et al.* (1972) studied baso-lateral membranes prepared from isolated rat intestinal epithelial cells and did not report fatty acid values, but did find somewhat higher lipid phosphate values than those described in the present paper. Forstner *et al.* (1968) found low amounts of non-esterified fatty acids (0.016 mg/mg of protein) in brush borders of rat intestine. Ockner & Manning (1976) did find relatively high concentrations of fatty acids ($0.3 \mu\text{mol}$ /mg of protein) in an intestinal microsomal fraction.

Re-esterification of fatty acids to triacylglycerols in the formation of very-low-density lipoproteins and chylomicrons has been thought previously to be a function of the intestinal smooth endoplasmic reticulum (Green & Glickman, 1981), but subsequent elegant electron-microscopic studies have suggested that, with low rates of fat absorption, accumulation of triacylglycerol occurs only in the Golgi (Marenus & Sstrand, 1982). Conceivably, the fatty acids in these Golgi fractions could be involved in triacylglycerol synthesis and packaging. Fat absorption and chylomicron or very-low-density-lipoprotein production have not been studied in vitamin D-deficiency.

The non-esterified fatty acids may also be a result of phospholipase activity such as is involved in the membrane-remodelling process that occurs extensively in Golgi (Morr *et al.*, 1979). Phospho-

lipase A₂ activity has been shown by O'Doherty (1979) to be increased by 1,25-dihydroxycholecalciferol (calcitriol) in rat duodenal homogenates 3 h after repletion of vitamin D-deficient animals. Lysophosphatidylcholine acyltransferase was also increased, and he postulated that these enzyme activities resulted in increased turnover and modification of the phospholipid composition of the epithelial cell. He did not study earlier times such as 15 min, where MacLaughlin *et al.* (1980) reported a change in Golgi Ca²⁺ uptake. Other work (reviewed by Rasmussen *et al.*, 1982) has shown that one of the actions of vitamin D is the modification of phospholipids in the brush border. Several intestinal phospholipases have been demonstrated (Mansbach *et al.*, 1982; Shakir *et al.*, 1982) that appear to be distinct from pancreatic lipase and may be involved in the formation of the membrane non-esterified fatty acids.

Fatty acids have not been implicated previously as a site for Ca²⁺ binding in other studies of Ca²⁺ uptake by intestinal membrane vesicles. Miller *et al.* (1982) studied Ca²⁺ binding to brush-border vesicles and found a binding site with similar affinity to the high-affinity site that we have found in Golgi. They ascribe this to a phosphoprotein, but their data would also be compatible with fatty acid binding. Ghijssen *et al.* (1982) have described ATP-dependent Ca²⁺ uptake in baso-lateral membrane vesicles that were prepared, like our fractions, by a modification of the method of Mircheff & Wright (1976). Their membranes would be expected to include the Golgi fractions studied in the present paper, yet they report very little Ca²⁺ binding. The use of Ca²⁺-EGTA buffers, La³⁺ in the Ca²⁺-uptake stop solution or the use of isolated cells as a starting point may be important in explaining this difference. It is difficult to speculate on the role of intestinal Golgi non-esterified fatty acids, and the relevance of the vitamin D-dependent change in their concentrations, until their sites of origin and their functions are determined.

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