Characterization of the vitamin D-dependent Ca^{2+} -binding sites in rat intestinal Golgi-enriched membrane fractions

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Rat intestinal Golgi-enriched membrane fractions take up Ca^{2+} by a vitamin D-dependent process that has been shown to recover within 15 min of repletion of vitamin D-deficient animals with intravenous 1,25-dihydroxycholecalciferol. The present paper reports studies characterizing the Ca^{2+} -binding sites of these membrane fractions. Equilibrium binding of Ca^{2+} at concentrations between 5 and 400 μM showed significant decreases at all concentrations in membranes derived from vitamin D-deficient animals when compared with normal control-diet-fed animals. The predominant class of binding sites had a relatively high affinity for Ca^{2+} (K_D approx. 3 μM). Vitamin D-deficiency did not change the affinity of this class of site, but decreased the number from 347 ± 26 to 168 ± 50 nmol of Ca^{2+} bound/mg of protein (means $\pm$ S.D.). Mg^{2+} inhibited binding only at low Ca^{2+} concentrations, and the characteristics of this binding suggested positive co-operativity between two binding sites. Equimolar concentrations of Zn^{2+} , La^{3+} , Pb^{2+} and Mn^{2+} inhibited Ca^{2+} binding by over 50%. Increased ionic strength decreased Ca^{2+} binding by no more than half. Binding was maximal at pH 7.5 and half-maximal at pH 6.3. The large number of binding sites with relatively high affinity for Ca^{2+} suggests that it is unlikely that this binding is to any specific protein or to non-specific sites present on many proteins, and that the most likely sites are lipid molecules.

The active metabolite of vitamin D, 1,25(OH) $_2$ D $_3$, regulates the intestinal absorption of calcium and phosphates (Lawson, 1978; Norman, 1980). Despite much research, the primary effects of 1,25(OH) $_2$ D $_3$ in controlling absorption remain unknown (Lawson, 1981). Many effects induced by 1,25(OH) $_2$ D $_3$ have been described in intestinal membranes (for review, see Nemere & Norman, 1982); these include changes in membrane-bound proteins such as alkaline phosphatase (Moriuchi & DeLuca, 1976), intestinal membrane Ca^{2+} -binding protein (Kowarski & Schachter, 1980) and Ca^{2+} -stimulated ATPase (Ghijsen & van Os, 1982). Other proteins affected by vitamin D include the cytoplasmic Ca^{2+} -binding protein (Wasserman, 1980) and the cytoskeletal protein actin (Wilson & Lawson, 1978). New protein synthesis, however, may not be necessary for 1,25(OH) $_2$ D $_3$ to produce its immediate effect on

Ca^{2+} transport, as this is not blocked by protein-synthesis inhibitors such as cycloheximide (Bikle *et al.*, 1978). Changes in membrane lipids in response to 1,25(OH) $_2$ D $_3$ have also been described and are reviewed by Rasmussen *et al.* (1982).

Membrane turnover, with synthesis of glycoproteins and phospholipids, takes place in the endoplasmic reticulum and Golgi. Changes may be seen in these membranes before they are expressed in the brush-border or baso-lateral membranes (Quaroni *et al.*, 1979). Additionally, vesicles derived from the intracellular membranes of the Golgi-endoplasmic-reticulum-lysosomal system may be responsible for transport of material through epithelia, and Ca^{2+} in the intestinal epithelial cell has been shown to be localized to such vesicles (Sampson *et al.*, 1970; Warner & Coleman, 1975; Davis *et al.*, 1979).

Rat intestinal Golgi-enriched membrane fractions take up Ca^{2+} in a vitamin D-dependent manner, as has been described by workers from our laboratory (Freedman *et al.*, 1977, 1981; MacLaughlin *et al.*, 1980). This uptake was shown

Abbreviations used: 1,25(OH) $_2$ D $_3$, 1,25-dihydroxycholecalciferol (calcitriol); Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

to recover in a biphasic manner within 15 min of intravenous repletion of vitamin D-deficient animals with $1,25(\text{OH})_2\text{D}_3$. We report in the present paper studies characterizing the sites responsible for Ca^{2+} uptake in normal non-deficient animals and also in those made vitamin D-deficient. We believe that this evidence suggests that lipid components, possibly non-esterified fatty acids, and not proteins, are the Ca^{2+} -binding sites involved in this vitamin D-dependent effect.

Experimental

Animals

Male weanling rats were obtained from Holtzman (Madison, WI, U.S.A.) and were fed on either a vitamin D-deficient diet or a control diet as described by Bloor *et al.* (1982). Animals between 5 and 8 weeks of age were starved for 16 h and then killed by cervical dislocation. Serum $[\text{Ca}^{2+}]$ at the time of death was significantly decreased in the vitamin D-deficient animals to $1.31 \pm 0.06 \text{ mM}$ (mean $\pm$ S.E.M.) from control values of $2.31 \pm 0.09 \text{ mM}$ ($P < 0.001$).

Membrane preparation

Golgi-enriched membrane fractions were prepared essentially as described previously by Freedman *et al.* (1977). Briefly, the entire small intestine was removed, washed with ice-cold 0.154 M-NaCl , everted and scraped to remove the epithelial cells. Homogenization (Dounce homogenizer; loose pestle, ten strokes; an initial centrifugation at $55000g$ for 20 min and then 70 further strokes of the resuspended pellet) was followed by differential centrifugation ($1500g$ for 15 min and $55000g$ for 45 min) to collect a crude membrane fraction. This was then separated by discontinuous sorbitol-density-gradient centrifugation. The least dense fractions (sorbitol at 20 and 30 g/dl and their interface, i.e. with an approximate density of $\leq 1.10 \text{ g/ml}$), were pooled and were shown to be enriched in Golgi membranes; similar purification for galactosyl-transferase activity was obtained to that found by Freedman *et al.* (1977). The membranes were washed in buffer containing 260 mM-sorbitol and $5 \text{ mM-imidazole/acetate}$, pH 7.5, centrifuged and resuspended in a similar buffer. Although assays were usually completed within 24 h of preparation of the membrane fractions, there was no change in Ca^{2+} uptake when the fractions were frozen and stored at -70°C . Protein was measured by the method of Bradford (1976) with bovine globulin as standard and reagent from Bio-Rad (Richmond, CA, U.S.A.).

Measurement of Ca^{2+} uptake

Membrane preparations were suspended at a protein concentration of $25 \mu\text{g/ml}$ in sor-

bitol/imidazole/acetate buffer as described above containing between 5 and $400 \mu\text{M-CaCl}_2$ and $0.2-0.4 \mu\text{Ci}$ of $^{45}\text{Ca}^{2+}/\text{ml}$ (Amersham, Arlington Heights, IL, U.S.A.). Free Ca^{2+} concentrations were confirmed with a Ca^{2+} -sensitive electrode (WPI Instruments, New Haven, CT, U.S.A.). After incubation at room temperature (usually 18°C), portions ($12.5 \mu\text{g}$ of protein) were collected by filtration on nitrocellulose filters ($0.45 \mu\text{m}$ pore size; BA85; Schleicher and Schuell, Keene, NH, U.S.A.) that had previously been soaked in 10 mM-CaCl_2 , and these were then washed with 5 ml of ice-cold Ca^{2+} -free buffer. The filters were dissolved and the radioactivity measured and corrected for binding to blank discs. For each animal, the value of Ca^{2+} uptake was the mean of at least two determinations.

Effects of various agents on Ca^{2+} uptake

The Ca^{2+} ionophore A23187 (Calbiochem-Behring, La Jolla, CA, U.S.A.) was dissolved in methanol and added at various concentrations to the membrane preparations before these were diluted and left overnight. The other cations studied (Mg^{2+} , Zn^{2+} , La^{3+} , Pb^{2+} , Ba^{2+} , Mn^{2+} , K^+ and Na^+) were added as their chlorides or acetates shortly before the addition of the Ca^{2+} -containing buffer to the membranes. The effects of ionic strength were investigated by washing and resuspending the membranes from the density gradient in solutions containing $5 \text{ mM-imidazole/acetate}$ buffer, pH 7.5, and $65 \text{ mM-KCl} + 130 \text{ mM-sorbitol}$, or 130 mM-KCl , or the usual 260 mM-sorbitol . The effects of pH were studied in buffers ranging in pH from 5 to 8.5 containing 5 mM-histidine and imidazole or acetate to buffer as appropriate. Neuraminidase (*Clostridium perfringens*; Sigma, St. Louis, MO, U.S.A.; 0.42 unit/mg of membrane protein) was incubated with the membranes at pH 5 and 37°C for 30 min, the pH adjusted to 7.5 with 1 M-imidazole and Ca^{2+} uptake measured as usual.

Statistical analysis

Mean Ca^{2+} uptake values from normal and vitamin D-deficient animals were compared by the unpaired Student's *t* test. Paired *t* tests were used for comparisons within the same animal. Computer line fitting to equilibrium binding data was by non-linear weighted least-squares minimization modified from the program described by Kohn *et al.* (1979).

Results

Kinetics of Ca^{2+} uptake

As described in previous studies, the Golgi-membrane-enriched fractions took up large

Table 1. Ca^{2+} uptake by intestinal Golgi membranes: effect of vitamin D deficiency

$[\text{Ca}^{2+}] = 0.4 \text{ mM}$. The number of animals in each group is shown in parentheses. Ca^{2+} -uptake values are expressed as means $\pm$ S.E.M. Statistical comparisons were performed by unpaired Student's *t* test.

Diet	Ca^{2+} uptake (nmol/mg of protein)		
	1 min	30 min	12 h
Control	116 $\pm$ 12 (30)	318 $\pm$ 17 (30)	546 $\pm$ 32 (25)
Vitamin D-deficient	79 $\pm$ 8 (19)	206 $\pm$ 12 (19)	343 $\pm$ 28 (16)
	$P < 0.05$	$P < 0.001$	$P < 0.001$

amounts of Ca^{2+} (Freedman *et al.*, 1977). When the sorbitol/imidazole/acetate buffer (pH 7.5) used in most of these studies and the Tris/Hepes/sorbitol buffer system used by Freedman *et al.* (1981) were compared by using membranes from the same animals, no significant difference in Ca^{2+} uptake was found. Vitamin D-deficient rats produced Golgi membranes that took up less Ca^{2+} than those from animals fed on the control vitamin D-sufficient diet (Table 1). The size of the difference was less than that found previously, but is nonetheless highly significant ($P < 0.001$ for uptake at 30 min or 12 h). Equilibrium values were reached between 6 h and 12 h (Fig. 1a) and were greater than the 30 min Ca^{2+} uptake by an average factor of 1.7 ± 0.1 .

Ca^{2+} uptake was not stimulated by the simultaneous addition of, or preincubation with, 1 mM-MgATP. A23187, when preincubated with the membrane fractions at concentrations up to 0.1 mM, produced no effect on the uptake of Ca^{2+} (Fig. 1b). Exchange of bound Ca^{2+} with free Ca^{2+} was measured by adding tracer radioactive Ca^{2+} to membranes pre-equilibrated with non-radioactive Ca^{2+} , and was shown to be slow (29% in the first 1 h), but virtually all of the Ca^{2+} bound at a $[\text{Ca}^{2+}]$ of 0.4 mM could be released within 1 min by the addition of 1 mM-EGTA.

Equilibrium Ca^{2+} binding

Ca^{2+} binding to Golgi membrane fractions was measured after a 12 h incubation. Fig. 2 shows the mean values obtained with total added Ca^{2+} concentrations between 5 and 400 μM ; vitamin D deficiency significantly decreased Golgi Ca^{2+} uptake at all these concentrations. Scatchard plots of these data (Fig. 3) suggest that in vitamin D deficiency there is a decreased number of Ca^{2+} -binding sites, but with similar affinity for Ca^{2+} to that in normal control-diet-fed animals. The results are better described by binding to two independent classes of sites rather than to one class of sites. Computerized fitting estimated the association constant (K_A) for the higher-affinity site in control animals as $(2.8 \pm 0.4) \times 10^5 \text{ M}^{-1}$ (mean $\pm$ S.D.) and for the lower-affinity site as $(6.7 \pm 3.8) \times 10^3 \text{ M}^{-1}$.

Fig. 1. Kinetics of Ca^{2+} uptake

(a) Time course of Ca^{2+} uptake by Golgi membranes prepared from a normal, control-diet-fed animal. Incubation was at room temperature (18°C). $[\text{Ca}^{2+}]$ was: $\bullet$, 400 μM ; $\blacktriangle$, 10 μM ; $\square$, 10 μM , with 1 mM- Mg^{2+} . Ca^{2+} uptake was determined at times up to 12 h. (b) Lack of effect of A23187 on Ca^{2+} binding to Golgi membranes prepared from a control animal. A23187 was preincubated at a concentration of 0.1 mM with undiluted membranes, and Ca^{2+} binding was measured at $[\text{Ca}^{2+}]$ of 10 μM and 400 μM . Symbols: $\circ$, $\square$, + A23187; $\bullet$, $\blacksquare$, no A23187.

Vitamin D-deficient animals were not significantly different. The higher-affinity site thus has a dissociation constant (K_D) of about $3\text{ }\mu\text{M}$. There was no significant difference in the estimated number of low-affinity binding sites, but the number of high-affinity sites was decreased in vitamin D-deficient rats from 347 ± 26 to $168 \pm 50\text{ nmol/mg}$ of protein ($P < 0.001$).

Fig. 2. Equilibrium Ca^{2+} uptake in Golgi-enriched membrane fractions

Incubation was for 12h with total $[\text{Ca}^{2+}]$ between 5 and $400\text{ }\mu\text{M}$. Each point is the mean $\pm$ S.E.M. for at least four, and usually six to ten animals. Ca^{2+} uptake in membranes prepared from control-diet-fed animals ($\bullet$) differed from vitamin D-deficient-diet-fed animals ($\circ$) at all concentrations ($P < 0.05$ or better).

Effect of other cations on equilibrium Ca^{2+} uptake

Equilibrium Ca^{2+} binding in the presence of 1 mM-Mg^{2+} is shown in Fig. 4 for the same range of Ca^{2+} concentrations. There was no significant decrease at a $[\text{Ca}^{2+}]$ of 0.4 mM , but at lower concentrations significantly decreased binding was seen in the presence of Mg^{2+} , and the Ca^{2+} -binding curve assumed a sigmoid shape. A Hill plot (Segel, 1975) of this curve gave a slope of 1.95, which suggests that in the presence of Mg^{2+} there is positive co-operativity between two Ca^{2+} -binding sites. Similar results were found in vitamin D-deficient and control animals.

Zn^{2+} and La^{3+} (both at 0.1 mM) inhibited equilibrium Ca^{2+} binding to a much greater extent than did 1 mM-Mg^{2+} , and their effect over a range of Ca^{2+} concentrations is also shown in Fig. 4. Other cations were also studied. Fig. 5 shows the decrease in Ca^{2+} binding produced by the addition of an equimolar concentration (0.1 mM) of competing cation. Zn^{2+} , La^{3+} , Pb^{2+} and Mn^{2+} all inhibited Ca^{2+} binding by over 50% ($P < 0.0005$) and, assuming that they bound at the same site, had a higher affinity than did Ca^{2+} . Ba^{2+} , Mg^{2+} , Na^{+} and K^{+} had much less effect in decreasing Ca^{2+} binding.

Increasing the ionic strength of the medium in which the Ca^{2+} uptake was determined also decreased binding. When the sorbitol in the usual buffer (260 mM) was decreased to 130 mM and replaced with 65 mM-KCl , equilibrium Ca^{2+} binding was decreased to 50% at a $[\text{Ca}^{2+}]$ of 0.1 mM , and to 81% at 0.4 mM ($P < 0.001$ for both).

Fig. 3. Scatchard plots of equilibrium Ca^{2+} binding to Golgi-enriched membrane fractions (a) Normal-diet-fed animals; (b) vitamin D-deficient-diet-fed animals. Each point represents the mean $\pm$ S.D. for at least four, and more usually six to ten animals. Protein concentration was $25\text{ }\mu\text{g/ml}$. The ordinate is Ca^{2+} bound (nmol/mg of protein) divided by the free Ca^{2+} concentration (i.e. total - bound Ca^{2+} in nmol/ml). Analysis of the data is described in the text.

Fig. 4. Equilibrium Ca^{2+} binding to Golgi-enriched membrane fractions in the presence of other cations

Membranes were prepared from normal-diet-fed animals and equilibrium Ca^{2+} binding measured at $[\text{Ca}^{2+}]$ 5–400 μM with no other cation (●), +1 mM- Mg^{2+} (□), +0.1 mM- Zn^{2+} (▲) or +0.1 mM- La^{3+} (▽). Each point represents the mean $\pm$ S.E.M. for at least four animals.

Fig. 6. Effect of pH on equilibrium Ca^{2+} binding. Equilibrium Ca^{2+} binding to Golgi-enriched membrane fractions was measured at different buffer pH values with 0.1 mM- Ca^{2+} . Each point is the mean $\pm$ S.E.M. for at least six animals of the percentage of the maximum binding at any pH. Half-maximal binding occurred at pH 6.3.

Fig. 5. Effect of equimolar concentrations of other cations on equilibrium Ca^{2+} binding to Golgi-enriched membrane fractions

Equilibrium Ca^{2+} binding was measured with a $[\text{Ca}^{2+}]$ of 0.1 mM alone and in the presence of each of the cations shown on the abscissa (also at 0.1 mM). Each column is the mean $\pm$ S.E.M. (shown by the bar) of the percentage of Ca^{2+} binding with no other added cations and represents seven animals.

Replacing all the sorbitol with 130 mM-KCl did not result in a significantly larger decrease in binding. Again, results for vitamin D-deficient and non-deficient animals were similar.

pH-dependence of Ca^{2+} binding

Equilibrium Ca^{2+} binding was measured at 0.4 mM- Ca^{2+} over a range of pH values from 5 to

8.5 (Fig. 6). Binding was usually maximal at pH 7.5, was significantly decreased at higher pH ($P < 0.001$ at pH 8.5), and no significant binding occurred at values more acidic than pH 6. Interpolation indicated half-maximal binding at a pH of 6.3, and this is presumed to represent the pK_a of the binding site. Results for vitamin D-deficient and normal animals were similar. When the membrane fractions were kept at pH 5 overnight and the pH then adjusted to 7.5, the Ca^{2+} binding was similar to that of preparations kept at pH 7.5, indicating that the decreased binding at pH 5 was reversible. The Ca^{2+} bound at pH 7.5 was almost completely released within 1 min by lowering the pH to 5.

Effect of neuraminidase treatment

No effect on Ca^{2+} binding was found when membrane preparations were pretreated with neuraminidase at pH 5 for 30 min, and Ca^{2+} uptake then measured as usual at pH 7.5.

Discussion

Our laboratory has previously produced results that indicated that there is a vitamin D-dependent change in rat intestinal membrane fractions enriched for the Golgi marker, galactosyltransferase activity (Freedman *et al.*, 1977). These

membrane fractions had a great capacity to bind Ca^{2+} , and MacLaughlin *et al.* (1980) showed that the decrease in Ca^{2+} uptake produced by vitamin D deficiency could be rapidly reversed in a biphasic manner by $1,25(\text{OH})_2\text{D}_3$. It was postulated that the Golgi change could represent membrane Ca^{2+} -transport components undergoing modification, or alternatively, a means of sequestration of Ca^{2+} . The purpose of the present studies was to clarify the nature of the vitamin D-dependent Ca^{2+} binding to Golgi-enriched membrane fractions and to determine how this was modified by the vitamin D status of the animal.

Vitamin D deficiency significantly decreased membrane equilibrium Ca^{2+} binding at all Ca^{2+} concentrations between 5 and 400 μM ; higher and lower concentrations were not studied. Lower Ca^{2+} concentrations are difficult to achieve accurately without the use of a Ca^{2+} -buffering system (Reed & Bygrave, 1975), and this may alter significantly the properties of some membrane systems (Waisman *et al.*, 1981; Kotagal *et al.*, 1982). The Ca^{2+} concentrations chosen approximate the range where active Ca^{2+} transport is most evident in duodenal gut-sacs (Schachter *et al.*, 1960). The earlier time points reported previously (Freedman *et al.*, 1981) may not have measured true equilibration, which was shown here to take at least 6 h. The buffering system contains no soluble anion (such as oxalate) that could precipitate an insoluble Ca^{2+} salt, and uptake occurs in the absence of an energy source such as ATP. Also, the Ca^{2+} ionophore does not affect the rate or amount of Ca^{2+} taken up. Thus, as discussed previously (Freedman *et al.*, 1981), the Ca^{2+} taken up must be bound to the membrane fractions or their contents.

Analysis of the equilibrium binding data indicates that the predominant vitamin D-dependent Ca^{2+} binding is to a class of sites with a K_D of about 3 μM and that this affinity is not significantly changed in the preparations from vitamin D-deficient animals. The binding sites with lower affinity do not show any vitamin D-dependent change in either number or affinity, and will not be discussed further. The dissociation constant for the high-affinity site is lower than values reported for Ca^{2+} binding to sialic acid (Jaques *et al.*, 1977), or to acidic amino acids, including γ -carboxyglutamic acid (Robertson *et al.*, 1978), but is similar to that for Ca^{2+} -lipid interactions (Seimiya & Ohki, 1973), and only slightly above that reported for Ca^{2+} -binding proteins such as intestinal cytosolic Ca^{2+} -binding protein (Ingersoll & Wasserman, 1971) and calmodulin (Klee *et al.*, 1980). Both these proteins are prevalent in the intestine, where calmodulin achieves some of its highest concentrations, but only intestinal Ca^{2+} -binding protein is decreased in vitamin D deficiency

(Thomasset *et al.*, 1981). Ca^{2+} -stimulated ATPase, sensitive to submicromolar Ca^{2+} concentrations, is present in intestinal baso-lateral membrane preparations, and its activity is decreased by vitamin D deficiency (Ghijsen *et al.*, 1980; Ghijsen & van Os, 1982). This protein could be an obvious candidate for the Ca^{2+} -binding site seen in the Golgi, particularly as other proteins (sucrase and amino-oligopeptidase) that are expressed on the intestinal cell-surface membrane have been located on Golgi endoplasmic-reticulum membranes during their synthesis (Hauri *et al.*, 1979; Ahnen *et al.*, 1983).

It is unlikely, however, that any of these proteins, or a similar protein, could be responsible for the magnitude of the Ca^{2+} -binding sites measured in these membrane fractions. There are several hundred nanomoles of Ca^{2+} bound per mg of total membrane protein, with about 200 nmol/mg of protein being vitamin D-dependent. Ca^{2+} -stimulated ATPase has an M_r of 115 000 (de Jonge *et al.*, 1981); even if all the protein in the membrane fractions were that ATPase, this would imply 20 molecules of Ca^{2+} bound to each protein molecule, an unlikely situation. Similarly, if the binding site were intestinal Ca^{2+} -binding protein (M_r about 10 000; Wasserman, 1980), all of the membrane protein would need to be composed of Ca^{2+} -binding protein to account for two molecules of Ca^{2+} bound to each molecule. This reasoning does not exclude the possibility that proteins, and new protein synthesis, are necessary for Ca^{2+} uptake by these membrane fractions (Dasmahapatra *et al.*, 1981); however, the site to which Ca^{2+} binds at equilibrium is unlikely to be a specific protein. The relatively high affinity for binding also effectively rules out non-specific sites present on a variety of glycoprotein molecules (for instance sialic acid or acidic amino acid residues). For these reasons, we considered whether the Ca^{2+} uptake could be binding to lipid molecules.

Several lipid groups will bind Ca^{2+} . Ca^{2+} -phospholipid interactions have been extensively studied; negatively charged phospholipids (phosphatidylserine, phosphatidylinositol and phosphatidic acid) may be involved in membrane fusion (Düzgünes *et al.*, 1980) and may act as Ca^{2+} ionophores (Tyson *et al.*, 1976). The apparent K_D for Ca^{2+} binding to phospholipids has been reported to be similar to that found here (Seimiya & Ohki, 1973), and total phospholipids are sufficiently abundant to account for the magnitude of binding. Vitamin D has been shown to affect phospholipids in the chick intestinal brush border (Rasmussen *et al.*, 1982); repleted chicks have an increased phosphatidylcholine/phosphatidylethanolamine ratio in their brush-border lipids and incorporate greater amounts of unsaturated fatty

acids in the phosphatidylcholine fraction (Matsumoto *et al.*, 1981). Also, increased activities of two enzymes responsible for phospholipid turnover, phospholipase A_2 and lysophosphatidylcholine acyltransferase, have been reported in duodenal homogenates from rats 3h after repletion with vitamin D (O'Doherty, 1979).

Changes in phospholipids are thus implicated in the action of vitamin D and are known to be involved in the action of several other humoral agents, including corticosteroids (Hirata & Axelrod, 1980; Nelson, 1980). Phospholipids, however, may not be directly responsible for the Ca^{2+} binding observed in our experiments. The pH-dependence of Ca^{2+} -phospholipid binding, measured in artificial lipid membranes, differs from that which we have observed. In our system, Ca^{2+} binding changed greatly between pH 6 and 7, with an apparent pK_a of 6.3; Ca^{2+} binding to phospholipids is relatively constant over this range (Seimiya & Ohki, 1973). Also, the effects of increasing ionic strength were not as great as in Ca^{2+} -phospholipid interactions (Ohki *et al.*, 1982).

Non-esterified fatty acids also bind Ca^{2+} with high affinity, and, when present in lipid membranes, have a pK_a similar to the 6.3 found here (Sobotka *et al.*, 1958; Ellis & Pauley, 1964; Rooney *et al.*, 1983). The fatty acid salts of bivalent ions (including Ca^{2+} , Mn^{2+} , Zn^{2+} and also La^{3+}) are poorly soluble, unlike those of Na^+ and K^+ . Non-esterified fatty acids in these membranes of the intestinal epithelial cell may be involved in the turnover and modification of membrane phospholipids and glycolipids, or in the absorption of fat and production of chylomicron particles.

We believe that the characteristics of the vitamin D-dependent Ca^{2+} binding to rat intestinal Golgi-enriched membrane fractions are most consistent with binding to non-esterified fatty acids. This hypothesis was investigated, and the results are presented in the following paper (Walters & Weiser, 1984).

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