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

Julian R. F. WALTERS and Milton M. WEISER
Division of Gastroenterology and Nutrition, State University of New York at Buffalo, and Buffalo General Hospital, 100 High Street, Buffalo, NY 14203, U.S.A.

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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 studies characterizing the Ca\(^{2+}\)-binding sites of these membrane fractions. Equilibrium binding of Ca\(^{2+}\) at concentrations between 5 and 400 \(\mu\)M 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 \(\mu\)M). Vitamin D-deficiency did not change the affinity of this class of site, but decreased the number from 347 \(\pm\) 26 to 168 \(\pm\) 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-ethanesulfonic acid.
to recover in a biphasic manner within 15 min of intravenous repletion of vitamin D-deficient animals with 1,25(OH)2D3. We report in the present paper studies characterizing the sites responsible for Ca2+ 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 Ca2+-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 Bloot et al. (1982). Animals between 5 and 8 weeks of age were starved for 16 h and then killed by cervical dislocation. Serum [Ca2+] at the time of death was significantly decreased in the vitamin D-deficient animals to 1.31 ± 0.06 mM (mean ± S.E.M.) from control values of 2.31 ± 0.09 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 55000 g for 20 min and then 70 further strokes of the resuspended pellet) was followed by differential centrifugation (1500 g for 15 min and 55000 g 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 ≤ 1.10 g/ml), were pooled and were shown to be enriched in Golgi membranes; similar purification for galactosyltransferase activity was obtained to that found by Freedman et al. (1977). The membranes were washed in buffer containing 260 mM sorbitol and 5 mM imidazole/acetate buffer, 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 Ca2+ 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 Ca2+ uptake

Membrane preparations were suspended at a protein concentration of 25 μg/ml in sorbitol/imidazole/acetate buffer as described above containing between 5 and 400 μM CaCl2 and 0.2—0.4 μCi of 45Ca2+/ml (Amersham, Arlington Heights, IL, U.S.A.). Free Ca2+ concentrations were confirmed with a Ca2+-sensitive electrode (WPI Instruments, New Haven, CT, U.S.A.). After incubation at room temperature (usually 18°C), portions (12.5 μg of protein) were collected by filtration on nitrocellulose filters (0.45 μm pore size; BA85; Schleicher and Schuell, Keene, NH, U.S.A.) that had previously been soaked in 10 mM CaCl2, and these were then washed with 5 ml of ice-cold Ca2+-free buffer. The filters were dissolved and the radioactivity measured and corrected for binding to blank discs. For each animal, the value of Ca2+ uptake was the mean of at least two determinations.

Effects of various agents on Ca2+ uptake

The Ca2+ 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 (Mg2+, Zn2+, La3+, Pb2+, Ba2+, Mn2+, K+ and Na+) were added as their chlorides or acetates shortly before the addition of the Ca2+-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 mM imidazole/acetate buffer, pH 7.5, and 65 mM CaCl2 + 130 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 Ca2+ uptake measured as usual.

Statistical analysis

Mean Ca2+ 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 Ca2+ uptake

As described in previous studies, the Golgi-membrane-enriched fractions took up large
Table 1. \( \text{Ca}^{2+} \) 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. \( \text{Ca}^{2+} \)-uptake values are expressed as means \pm S.E.M. Statistical comparisons were performed by unpaired Student's \( t \) test.

<table>
<thead>
<tr>
<th>Diet</th>
<th>1 min</th>
<th>30 min</th>
<th>12h</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>116 \pm 12 \ (30)</td>
<td>318 \pm 17 \ (30)</td>
<td>546 \pm 32 \ (25)</td>
</tr>
<tr>
<td>Vitamin D-deficient</td>
<td>79 \pm 8 \ (19)</td>
<td>206 \pm 12 \ (19)</td>
<td>343 \pm 28 \ (16)</td>
</tr>
<tr>
<td></td>
<td>( P &lt; 0.05 )</td>
<td>( P &lt; 0.001 )</td>
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amounts of \( \text{Ca}^{2+} \) (Freedman et al., 1977). When the sorbitol/imidazole/acetate buffer (pH 7.5) used in most of the 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 \( \text{Ca}^{2+} \) uptake was found. Vitamin D-deficient rats produced Golgi membranes that took up less \( \text{Ca}^{2+} \) 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 12h). Equilibrium values were reached between 6h and 12h (Fig. 1a) and were greater than the 30 min \( \text{Ca}^{2+} \) uptake by an average factor of 1.7 \pm 0.1.

\( \text{Ca}^{2+} \) 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 \( \text{Ca}^{2+} \) (Fig. 1b). Exchange of bound \( \text{Ca}^{2+} \) with free \( \text{Ca}^{2+} \) was measured by adding tracer radioactive \( \text{Ca}^{2+} \) to membranes pre-equilibrated with non-radioactive \( \text{Ca}^{2+} \), and was shown to be slow (29% in the first 1h), but virtually all of the \( \text{Ca}^{2+} \) bound at a \([\text{Ca}^{2+}] = 0.4 \text{ mM} \) could be released within 1 min by the addition of 1 mM-EGTA.

### Equilibrium \( \text{Ca}^{2+} \) binding

\( \text{Ca}^{2+} \) binding to Golgi membrane fractions was measured after a 12h incubation. Fig. 2 shows the mean values obtained with total added \( \text{Ca}^{2+} \) concentrations between 5 and 400 \( \mu \text{M} \); vitamin D deficiency significantly decreased Golgi \( \text{Ca}^{2+} \) uptake at all these concentrations. Scatchard plots of these data (Fig. 3) suggest that in vitamin D deficiency there is a decreased number of \( \text{Ca}^{2+} \)-binding sites, but with similar affinity for \( \text{Ca}^{2+} \) 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 \((2.8 \pm 0.4) \times 10^4 \text{ M}^{-1} \) (mean \pm S.D.) and for the lower-affinity site as \((6.7 \pm 3.8) \times 10^3 \text{ M}^{-1} \).

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**Fig. 1.** Kinetics of \( \text{Ca}^{2+} \) uptake

(a) Time course of \( \text{Ca}^{2+} \) uptake by Golgi membranes prepared from a normal, control-diet-fed animal. Incubation was at room temperature (18°C). \([\text{Ca}^{2+}] \) was: \( \bullet, \ 400 \mu \text{M}; \ ▲, \ 10 \mu \text{M}; \ □, \ 10 \mu \text{M}, \) with 1 mM-Mg\( \text{ATP} \). \( \text{Ca}^{2+} \) uptake was determined at times up to 12h. (b) Lack of effect of A23187 on \( \text{Ca}^{2+} \) binding to Golgi membranes prepared from a control animal. A23187 was preincubated at a concentration of 0.1 mM with undiluted membranes, and \( \text{Ca}^{2+} \) binding was measured at \([\text{Ca}^{2+}] \) of 10 \( \mu \text{M} \) and 400 \( \mu \text{M} \). Symbols: \( \bigcirc, \ □, + \text{A23187}; \ ●, ▲, \) no A23187.
Vitamin D-deficient animals were not significantly different. The higher-affinity site thus has a dissociation constant ($K_D$) of about 3 μM. 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 ± 50 nmol/mg of protein ($P < 0.001$).

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 [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 [Ca$^{2+}$] of 0.1 mM, and to 81% at 0.4 mM ($P < 0.001$ for both).
pH-dependence of intestinal Golgi Ca\textsuperscript{2+} binding

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

Membranes were prepared from normal-diet-fed animals and equilibrium Ca\textsuperscript{2+} binding measured at [Ca\textsuperscript{2+}] 5-400 \textmu M with no other cation (●), +1 mm-Mg\textsuperscript{2+} (□), +0.1 mm-Zn\textsuperscript{2+} (△) or +0.1 mm-La\textsuperscript{3+} (◇). Each point represents the mean ± S.E.M. for at least four 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\textsuperscript{2+} binding

Equilibrium Ca\textsuperscript{2+} binding was measured at 0.4 mm-Ca\textsuperscript{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\textsubscript{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\textsuperscript{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\textsuperscript{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\textsuperscript{2+} binding was found when membrane preparations were pretreated with neuraminidase at pH 5 for 30 min, and Ca\textsuperscript{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\textsuperscript{2+}, and MacLaughlin et al. (1980) showed that
the decrease in Ca\textsuperscript{2+} uptake produced by vitamin
D deficiency could be rapidly reversed in a biphasic manner by 1,25(OH)\textsubscript{2}D\textsubscript{3}. It was postu-
lated that the Golgi change could represent mem-
brane Ca\textsuperscript{2+}-transport components undergoing modifi-
cation, or alternatively, a means of seque-
stration of Ca\textsuperscript{2+}. The purpose of the present
studies was to clarify the nature of the vitamin D-
dependent Ca\textsuperscript{2+} binding to Golgi-enriched mem-
brane fractions and to determine how this was
modified by the vitamin D status of the animal.

Vitamin D deficiency significantly decreased
membrane equilibrium Ca\textsuperscript{2+} binding at all Ca\textsuperscript{2+}
concentrations between 5 and 400 \(\mu\)M; higher and
lower concentrations were not studied. Lower Ca\textsuperscript{2+}
concentrations are difficult to achieve accurately without the use of a Ca\textsuperscript{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\textsuperscript{2+} concentrations chosen approxi-
mate the range where active Ca\textsuperscript{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
tue equilibration, which was shown here to take at
least 6h. The buffering system contains no soluble
anion (such as oxalate) that could precipitate an
insoluble Ca\textsuperscript{2+} salt, and uptake occurs in the
absence of an energy source such as ATP. Also, the
Ca\textsuperscript{2+} ionophore does not affect the rate or amount
of Ca\textsuperscript{2+} taken up. Thus, as discussed previously
(Freedman et al., 1981), the Ca\textsuperscript{2+} taken up must be
bound to the membrane fractions or their contents.

Analysis of the equilibrium binding data indi-
cates that the predominant vitamin D-dependent
Ca\textsuperscript{2+} binding is to a class of sites with a \(K_D\) of
about 3 \(\mu\)M 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\textsuperscript{2+} binding to sialic acid (Jaques et al., 1977), or
to acidic amino acids, including \(\gamma\)-carbox-
ylglutamic acid (Robertson et al., 1978), but is
similar to that for Ca\textsuperscript{2+}-lipid interactions (Sei-
miya & Ohki, 1973), and only slightly above that
reported for Ca\textsuperscript{2+}-binding proteins such as intesti-
nal cytosolic Ca\textsuperscript{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\textsuperscript{2+}-
binding protein is decreased in vitamin D defic-
iency (Thomasset et al., 1981). Ca\textsuperscript{2+}-stimulated
ATPase, sensitive to submicromolar Ca\textsuperscript{2+} concen-
trations, is present in intestinal baso-lateral mem-
brane 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\textsuperscript{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 locat-
ed 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\textsuperscript{2+}-binding sites
measured in these membrane fractions. There are
several hundred nanomoles of Ca\textsuperscript{2+} bound per mg
of total membrane protein, with about 200 nmol/mg
of protein being vitamin D-dependent.
Ca\textsuperscript{2+}-stimulated ATPase has an \(M_r\) of 115000
(de Jonge et al., 1981); even if all the protein in the
membrane fractions were that ATPase, this would
imply 20 molecules of Ca\textsuperscript{2+} bound to each protein
molecule, an unlikely situation. Similarly, if the
binding site were intestinal Ca\textsuperscript{2+}-binding protein
(\(M_r\) about 10000; Wasserman, 1980), all of the
membrane protein would need to be composed of
Ca\textsuperscript{2+}-binding protein to account for two molecules
of Ca\textsuperscript{2+} bound to each molecule. This reasoning
does not exclude the possibility that proteins, and
new protein synthesis, are necessary for Ca\textsuperscript{2+}
uptake by these membrane fractions (Dasmahapat-
tra et al., 1981); however, the site to which Ca\textsuperscript{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\textsuperscript{2+} uptake
could be bound to lipid molecules.

Several lipid groups will bind Ca\textsuperscript{2+}. Ca\textsuperscript{2+}-
phospholipid interactions have been extensively
studied; negatively charged phospholipids
(phosphatidylserine, phosphatidylinositol and
phosphatidic acid) may be involved in membrane
fusion (Düzgünès et al., 1980) and may act as Ca\textsuperscript{2+}
ionophores (Tyson et al., 1976). The apparent \(K_D\)
for Ca\textsuperscript{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/phosphatidyl-
ethanolamine ratio in their brush-border lipids and
incorporate greater amounts of unsaturated fatty
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acids in the phosphatidylcholine fraction (Matsumoto et al., 1981). Also, increased activities of two enzymes responsible for phospholipid turnover, phospholipase A\textsubscript{2} and lysophosphatidylcholine acyltransferase, have been reported in duodenal homogenates from rats 3 h 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\textsuperscript{2+} binding observed in our experiments. The pH-dependence of Ca\textsuperscript{2+}-phospholipid binding, measured in artificial lipid membranes, differs from that which we have observed. In our system, Ca\textsuperscript{2+} binding changed greatly between pH 6 and 7, with an apparent pK\textsubscript{a} of 6.3; Ca\textsuperscript{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\textsuperscript{2+}-phospholipid interactions (Ohki et al., 1982).

Non-esterified fatty acids also bind Ca\textsuperscript{2+} with high affinity, and, when present in lipid membranes, have a pK\textsubscript{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\textsuperscript{2+}, Mn\textsuperscript{2+}, Zn\textsuperscript{2+} and also La\textsuperscript{3+}) are poorly soluble, unlike those of Na\textsuperscript{+} and K\textsuperscript{+}. 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\textsuperscript{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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