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Decrease by cycloheximide of calcium binding and nonesterified fatty acids in rat-intestinal Golgi-enriched membrane fractions

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Rat intestinal Golgi-enriched membrane fractions bind more Ca^{2+} than do basolateral and microvillus-enriched membrane fractions, and this uptake is reduced by vitamin D-deficiency. The effect of the protein synthesis inhibitor, cycloheximide, on this Ca^{2+} binding was determined in rats fed a normal, vitamin D-sufficient diet. Cycloheximide, 1.5 mg/kg, rapidly reduced protein synthesis (measured by [^{3}H]leucine incorporation) to 12% of control values within 15 min, but Ca^{2+} binding diminished gradually to 50% of control values by 60 min. Ca^{2+} transport across gut sacs was also decreased. The reduction in Ca^{2+} binding was not due to an alteration in vesicle morphology or to a direct effect of cycloheximide. Nonesterified (free) fatty acids, the probable binding sites for Ca^{2+} in these membrane fractions, were reduced by cycloheximide to 48% of control values by 60 min. There was no significant change in total lipid phosphate. Cycloheximide may affect the synthesis of proteins necessary for the presence of nonesterified fatty acids in these Golgi membranes.

Introduction

Absorption of calcium by active transport in the small intestine involves translocation across the microvillus membrane, sequestration and movement through the cell, and extrusion across the basolateral membrane. These processes, and the way they are affected by the vitamin D metabolite, 1,25-dihydroxycholecalciferol, have been recently reviewed by several authors [1–3]. However, the method by which $1,25(OH)_2D_3$ increases calcium transport is debatable, as are the relative contributions of lipid changes (reviewed in Ref. 4) and new protein synthesis in the stimulation by $1,25(OH)_2D_3$. Also uncertain is the role of sequestration of calcium within subcellular organelles during absorption.

Electron probe microanalysis has shown concentrations of calcium in Golgi-rich supranuclear areas of intestinal epithelial cells; in vitamin D-deficient animals, these areas concentrate less calcium [5]. Workers from our laboratory have shown that rat intestinal membrane vesicle fractions enriched for galactosyltransferase activity, a Golgi membrane marker, have an initial rate and equilibrium level of Ca^{2+} uptake that is greater than that seen for vesicles enriched for microvillus or basolateral plasma membrane markers [6–8]. Furthermore, Ca^{2+} uptake has been shown to be significantly reduced in Golgi-enriched membrane fractions

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Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid.

prepared from vitamin D-deficient animals. Restoration of Golgi Ca^{2+} uptake by $1,25(OH)_2D_3$ was shown to precede the recovery of Ca^{2+} transport across gut sacs [7]. This uptake of Ca^{2+} was shown to represent binding and was not ATP-dependent [9].

Recent studies of the Ca^{2+}-binding sites in these Golgi-enriched membrane fractions indicated that they are likely to be nonesterified ('free') fatty acids which are present in unusually high concentrations compared to other membranes [10]. The origin of these fatty acids and their physiological role is not yet known, but they could represent a means of intracellular sequestration of Ca^{2+} concentrated within the intestinal Golgi apparatus by an active calcium pump. The aim of the present study was to determine the effect of protein synthesis inhibition by cycloheximide on Ca^{2+} uptake [11] and on the concentrations of nonesterified fatty acids in the Golgi fractions. Both are reduced by cycloheximide within 60 min, suggesting that there may be a relatively rapid turnover of the fatty acids in these membranes.

Materials and Methods

Weanling albino rats (Holtzman, Madison, WI) were maintained on a powdered control diet, (TD 79095, Teklad Test Diets, Madison, WI) containing 0.53% (w/w) Ca^{2+} and 2000 IU vitamin D-3/kg. The animals used were between 4 and 9 weeks of age. At varying times before being killed, they were injected intraperitoneally with either cycloheximide (1.5 mg/kg body wt., dissolved in 0.154 M NaCl) or 0.154 M NaCl alone. Each animal also received 50 μCi (approx. 1 nmol) of [4,5(n)-^{3}H]leucine (New England Nuclear Corp., Boston, MA; specific activity 47 Ci/mmol; pH 7), via tail vein 10 or 20 min before death.

Protein synthesis was measured as the proportion of [^{3}H]leucine radioactivity in a homogenate of mucosal scrapings that was precipitable by trichloroacetic acid. The precipitate was collected by filtration through glass fiber filters (Whatman's grade 934AH). Both precipitate and an aliquot of the original homogenate were digested with Protosol (New England Nuclear) at 50°C for 30 min and the radioactivity measured by liquid scintillation counting after the addition of OCS (Amersham). Protein was measured by the dye-binding method of Bradford [12] with bovine globulin standard and reagent from Bio-Rad (Richmond, CA.)

Golgi-enriched membrane fractions were prepared as previously described by our laboratory [6] using mucosal scrapings of the entire small intestine. Following homogenization and differential centrifugation, membranes were separated by sorbitol density gradient centrifugation. The membranes which layered at the interface between the 20 and 30 g/dl sorbitol fractions and those in the 30 g/dl fraction were collected, diluted with 260 mM sorbitol/20 mM Hepes-Tris buffer (pH 7) and centrifuged at $80\,000 \times g$ for 2 h. This pellet was resuspended in the same buffer by passing through a 26 gauge needle. The membranes in this fraction were similar to those described previously, and were enriched 5–8-fold for the Golgi marker, galactosyltransferase activity, and less than 4-fold for the basolateral membrane marker ($Na^+ + K^+$)-dependent ATPase [6,13].

Ca^{2+} uptake was measured within 24 h of completion of membrane preparation essentially as described before [10]. The incubation medium comprised 260 mM sorbitol/20 mM Tris-Hepes (pH 8)/0.4 mM $^{45}CaCl_2$ (New England Nuclear; specific activity, 1 Ci/mol). At various time intervals, aliquots were removed and immediately filtered through nitrocellulose filters (Schleicher & Schuell, BA85, 25 mm diameter, 0.45 μm) which had been presoaked in 10 mM $CaCl_2$. The filters were rinsed with 5 ml Ca^{2+}-free medium and dissolved in ACS (Amersham), and the radioactivity was measured.

The direct effect of cycloheximide on Ca^{2+} uptake was measured by incubating membrane fractions with $^{45}CaCl_2$ and cycloheximide concentrations of 0.1, 1 and 10 ng/mg protein. The concentration of cycloheximide present in the cytosol was estimated to be 1 ng/mg protein assuming that the intraperitoneal dose was distributed uniformly in all tissues.

Calcium transport across everted gut sacs was measured by the method of Martin and DeLuca [14]. The results were expressed as the ratio of $^{45}Ca^{2+}$ cpm in equal volumes of the serosal and mucosal solutions (S/M ratio).

Golgi-enriched membrane fractions from nor-

mal and cycloheximide treated rats were examined by electron microscopy. Fractions were fixed in buffer containing 4% glutaraldehyde, rinsed, and postfixed in sodium phosphate buffered 1% osmium tetroxide for 2 h. The membranes were then dehydrated and embedded, and thin sections were cut, post-stained with uranyl acetate and lead citrate and observed on a Siemens Elmiskop 1 A electron microscope at 80 kV.

Nonesterified fatty acids and phospholipids in the membrane fractions were extracted and measured as described before [10]. Briefly, lipids were extracted with methanol/chloroform [15], and separated by thin layer chromatography using a solvent system of petroleum ether/diethyl ether/acetic acid (90:10:1). The fatty acid and triacylglycerol spots were scraped off the chromatogram and quantified by dichromate oxidation [16]. Phospholipids were measured as total lipid phosphate [17].

Statistical analysis of data was by Student's unpaired *t*-test. Results are expressed as means ± S.E.

Results

In the intestinal mucosal scrapings obtained from normal untreated rats, 67.0 ± 2.4% of the [^{3}H]leucine counts were precipitated by 5% trichloroacetic acid. In rats given cycloheximide 15–80 min before death, the acid-precipitable radioactivity was greatly reduced (Table I). The non-precipitable radioactivity in the mucosal homogenate was not significantly different in the cycloheximide-treated animals; this indicated that uptake of leucine from the blood into the cytosolic pool, and thus the specific activity of leucine, was unchanged [18]. By these criteria, cycloheximide at a dose of 1.5 mg/kg body weight was shown to inhibit protein synthesis to 12% of control values within 15 min with continued suppression to at least 80 min.

TABLE I

INHIBITION OF INTESTINAL PROTEIN SYNTHESIS BY CYCLOHEXIMIDE

n = number of different animals; protein synthesis = ([^{3}H]Leu cpm in acid-precipitate/[^{3}H]Leu cpm in total homogenate)× 100.

Time after cycloheximide (min)	*n*	Protein synthesis	% of control values
Control	9	67 ± 2	100 ± 3
15	2	8 ± 1	12 ± 1
30	5	15 ± 2	22 ± 3
60	4	15 ± 4	22 ± 6
80	3	17 ± 2	25 ± 3

Ca^{2+} uptake by the Golgi-enriched membrane fractions from untreated animals was 331 ± 23 nmol/mg protein after 15 min incubation; this is similar to the values reported before [11,12]. The effect of in vivo administration of cycloheximide on the time-course of Ca^{2+} uptake is shown in Fig. 1. A progressive decrease in Ca^{2+} uptake occurred; the 15 min uptake values, which approximate to equilibrium, were statistically lower than control values in animals given cycloheximide 30 min or longer before death (Table II). The rates of onset of the effects of cycloheximide on [^{3}H]leucine incorporation and Golgi Ca^{2+} uptake are shown in Fig. 2. Although protein synthesis was maximally inhibited within 15 min of cycloheximide administration, the decline in Ca^{2+} uptake by

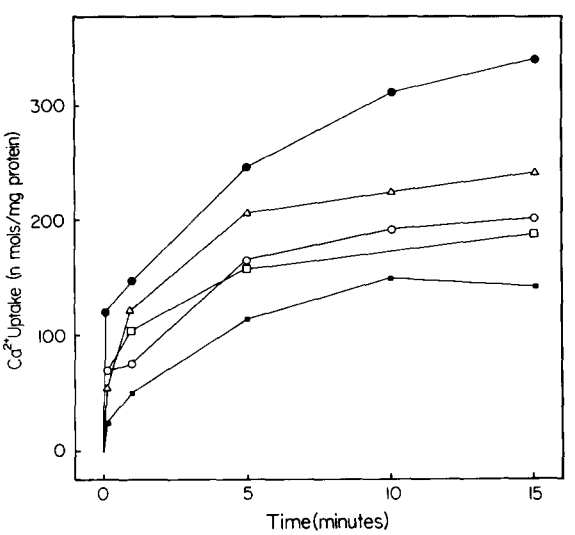

Fig. 1. Time-course of in vitro Ca^{2+} uptake by Golgi-enriched membrane fractions prepared from normal or cycloheximide-treated animals. Cycloheximide (1.5 mg/kg body wt.) was given 15 min (△), 30 min (○), 60 min (□) or 80 min (■) before starting the membrane preparation. ●, animals that received a control injection only. Ca^{2+} concentration in the assay was 0.4 mM. Points are mean values of 2–10 animals.

TABLE II

EFFECT OF CYCLOHEXIMIDE ON CALCIUM UPTAKE BY RAT-INTESTINAL GOLGI-ENRICHED MEMBRANE FRACTIONS

n = number of different animals. Calcium uptake was measured at $[Ca^{2+}] = 0.4$ mM and is expressed as nmol per mg protein/15 min. The statistical comparisons are with control values of Ca^{2+} uptake.

Time after cycloheximide (min)	n	Calcium uptake	% of control values	P
Control	7	331 ± 23	100 ± 7	–
30	6	203 ± 33	61 ± 10	< 0.01
60	7	167 ± 23	50 ± 7	< 0.001
80	3	142 ± 18	43 ± 5	< 0.001

Golgi-enriched membrane fractions was more gradual, decreasing steadily to 80 min after cycloheximide administration (Fig. 2). In these Golgi membrane vesicles 80 min after cycloheximide, both the time-course and amount of Ca^{2+} uptake resemble those seen with vitamin D-deficient animals [6].

To exclude a direct effect of cycloheximide on Golgi membrane vesicles, membranes prepared from normal, untreated rats were incubated in vitro with cycloheximide at concentrations of 0.1, 1.0 and 10.0 ng/ml. There was no effect on Ca^{2+} uptake at 1, 5, 15 or 30 min.

Ca^{2+} transport by everted duodenal sacs prepared from normal animals resulted in an S/M ratio of 3.5 ± 0.2 ($n = 7$) after 90 min incubation with 0.25 mM $CaCl_2$. In sacs made from rats that had received cycloheximide 60 min prior to death, Ca^{2+} transport was significantly reduced (S/M ratio, 2.4 ± 0.2; $n = 6$; $P < 0.01$).

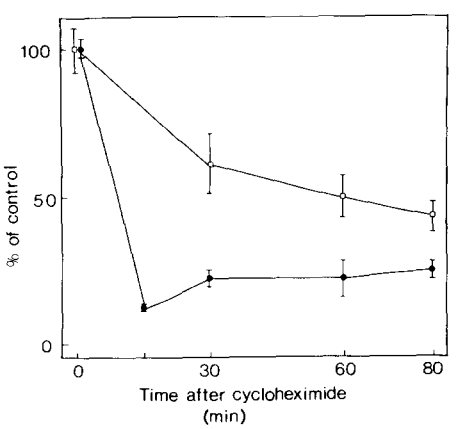

Fig. 2. Comparison of the time-course of inhibition by cycloheximide of intestinal protein synthesis and Ca^{2+} uptake. Points are mean ± S.E. of values expressed as percentages of the values found in control untreated animals. □, Ca^{2+} uptake; ●, protein synthesis. Animals received cycloheximide 15–80 min before death.

Electron micrographs of Golgi-enriched membrane fractions from normal and cycloheximide treated rats are shown in Fig. 3. Cycloheximide was given 60 min before death. There appears to be no difference in the size or number of closed vesicles or lipid droplets, or in the density of staining.

Nonesterified fatty acids were significantly reduced in concentration in lipid extracts of membrane fractions prepared from animals given cycloheximide 60 min before death, when compared with normal animals [10] (Table III). The reduction in the levels of nonesterified fatty acids to 48 ± 4% of control is similar to the reduction in Ca^{2+} uptake at this time (50 ± 7%). Phospholipid concentrations were not statistically different and

TABLE III

EFFECT OF CYCLOHEXIMIDE ON LIPIDS IN GOLGI-ENRICHED MEMBRANE FRACTIONS

Cycloheximide was given 60 min before death.

Treatment	n	Non-esterified fatty acids (μmol per mg protein)	Total lipid phosphate (μmol per mg protein)
Control	6	1.37 ± 0.12	0.63 ± 0.05
Cycloheximide	3	0.66 ± 0.05	0.62 ± 0.01
P		< 0.01	> 0.8

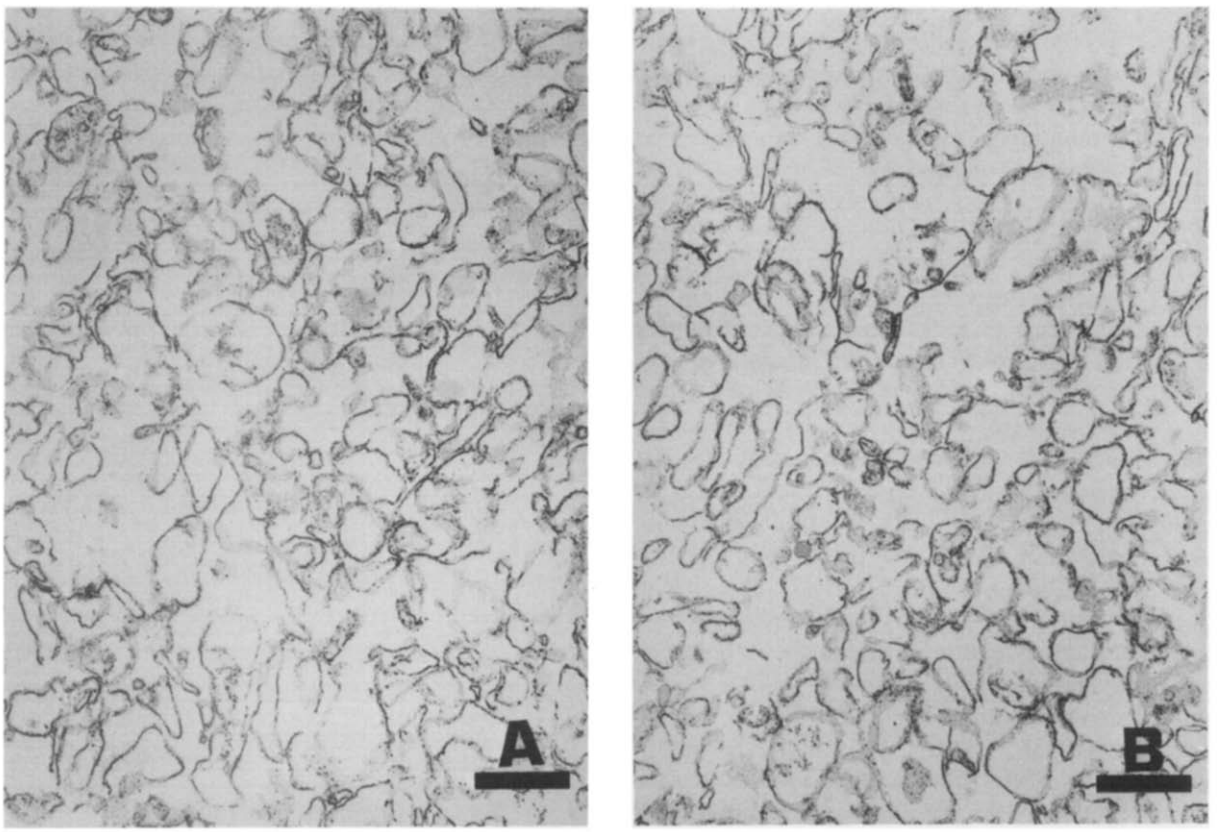

Fig. 3. Electron micrographs of Golgi-enriched membrane fractions prepared from (A) normal animals and (B) animals treated with cycloheximide 60 min before death. The bar represents 0.5 μm.

triacylglycerols were undetectable in Golgi prepared from cycloheximide-treated animals; thus there is no evidence that breakdown in Golgi of either of these groups of lipids is responsible for the formation of the nonesterified fatty acids.

Discussion

The main findings in this paper are that cycloheximide treatment will decrease both the concentration of nonesterified fatty acids present in rat-intestinal Golgi-enriched membrane fractions and the capacity of these membranes to take up and bind calcium. In these respects, though possibly by quite different mechanisms, cycloheximide treatment mimics the changes seen in vitamin D-deficiency, i.e., significant decreases in the unusually high levels of nonesterified fatty acids found in these fractions in normal animals [10]. As discussed previously [10], the physiological role of these fatty acids is not known, but they could be involved in fatty absorption and chylomicron synthesis by the intestinal Golgi membranes [19], or be the result of phospholipase activity related to membrane remodelling and modification [20].

Cycloheximide inhibits protein synthesis by blocking mRNA translation, and so will affect most cellular processes in which the synthesis and turnover of key enzymes is rapid. As judged by [^{3}H]leucine incorporation into acid-precipitable material, inhibition of protein synthesis occurred within 15 min in our experiments. The decline seen in Golgi nonesterified fatty acids suggests that they have a relatively high turnover rate, with a half-life in the membranes of about 60 min. As protein synthesis inhibition was not instantaneous

and was never more than 88%, the turnover rate may in fact be even more rapid. Possible candidates for the key protein(s) affected include phospho- and other lipases, and proteins such as cytosolic fatty-acid binding protein [21] (which may be involved in delivering fatty acids to the Golgi). Further speculation is difficult until more is known about the origin of the nonesterified fatty acids.

Other authors have reported the effects of cycloheximide and other inhibitors of protein synthesis on intestinal Ca^{2+} transport, but unlike our studies, these concerned vitamin D-deficient animals receiving repletion doses of vitamin D or its metabolites, and were designed to determine whether the mechanism by which vitamin D restored intestinal calcium transport required new protein synthesis. Bikle et al. [22] found that cycloheximide failed to inhibit the increase in Ca^{2+} transport induced by $1,25(OH)_2D_3$ in chick duodenum and found a moderate stimulation by cycloheximide of uninduced transport. A possible reason for the disparity with our results, which showed in normal animals an inhibition by cycloheximide, is that they used a Ca^{2+} concentration of 3 mM and were consequently studying absorption other than by the vitamin D-dependent pathway which saturates above 0.4 mM [23]. Franceschi and DeLuca [24] have shown that cycloheximide reversibly inhibits $1,25(OH)_2D_3$-dependent increases in Ca^{2+} uptake by cultured embryonic chick duodenum.

Of particular relevance to our findings is the morphological study by Morrissey et al. [25] which showed an inhibition by cycloheximide of $1,25(OH)_2D_3$-stimulated mineral vesicles present in chick intestinal epithelial cells. These mineralized vesicles are probably similar to those described by Warner and Coleman [5] and may be derived from or purify with, our Golgi-enriched membrane fraction. Along with mitochondria, these membrane vesicles could represent a means of sequestration of intracellular calcium, and nonesterified fatty acids may be the binding site for calcium.

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