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INTESTINAL BRUSH BORDER HYDROLASE TOPOGRAPHY

EFFECTS OF VITAMIN D-3 AND FILIPIN *

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Intestinal brush borders were isolated from vitamin D-3-treated and vitamin D-deficient chicks, and protein topography in the paired preparations assessed by the enzymatic release of four marker hydrolases. Exposure of the brush borders to the protease bromelain resulted in soluble levels of alkaline phosphatase, leucine aminopeptidase, maltase, and sucrase activities from preparations of vitamin D-3-treated birds that were 42%, 75%, 64%, and 56%, respectively, of corresponding activities released in preparations from rachitic chicks. Analyses for recovery of enzyme activity revealed that bromelain treatment selectively inactivated 43% of the alkaline phosphatase activity of brush borders obtained from vitamin D-3-replete birds, and preferentially diminished recovered sucrase activity in preparations from vitamin D-deficient chicks. In additional experiments, brush borders isolated from rachitic birds were treated in vitro with the polyene antibiotic filipin or an equivalent volume of vehicle. Subsequent exposure of such preparations to bromelain resulted in little or no differences in levels of marker hydrolase specific activities released from filipin- or vehicle-treated brush borders. However, analyses of membrane-bound specific activities after treatment of brush border preparations with a range of filipin concentrations, revealed a biphasic inhibition of approx. 30% for both maltase and sucrase, relative to vehicle controls, and a smaller effect on alkaline phosphatase and leucine aminopeptidase.

Introduction

We have recently reported that vitamin D mediates topographical alterations of chick intestinal brush border proteins, as judged by the technique of limited proteolysis [1]. Papain, the proteinase used in these studies, efficiently released three brush border marker hydrolases that retained full catalytic activity [1]. However, papain treatment of isolated brush borders resulted in minimal solubilization of alkaline phosphatase activity.

It is well known that changes in the activity of alkaline phosphatase, as well as its distribution, are intimately related to developmental stages [2], as well as hormonal stimulation of the intestinal epithelium. [3–5]. In order to determine whether the vitamin D-dependent increase in alkaline phosphatase activity of intestinal brush borders [3] is accompanied by topographical alterations, the previous studies were extended to monitor bromelain-mediated release of four marker hydrolase activities. The protease bromelain has been found to completely remove a number of alkaline

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phosphatase isoenzymes from HeLa cell surfaces [6].

In the present work, additional experiments were performed on isolated brush borders treated with filipin in vitro. The polyene antibiotic, filipin, under in vitro conditions stimulates intestinal calcium transport in a manner similar to that of vitamin D [7]. Moreover, filipin is known to bind cholesterol [8], a major constituent of brush border membrane lipids [9]. Thus, studies were undertaken to determine whether exposure of brush borders to the polyene antibiotic altered the topography of membrane-bound proteins, by the criterion of cleavage and release of marker hydrolase activities.

Materials and Methods

Animals and isolation of intestinal brush borders. Preparation of rachitic chicks and subsequent treatment with vitamin D-3 or vehicle were as previously described [1]. Methods for the isolation of intestinal brush borders [9] and their subsequent processing [1] have appeared elsewhere.

Limited proteolysis. Brush borders isolated from vitamin D-3-treated and -deficient chicks, were resuspended to 8 mg protein per ml of 50 mM sodium phosphate buffer (pH 6.0). A portion of each suspension was held on ice for reference rather than co-incubated with the treated aliquots, since pilot studies demonstrated no major perturbations in redistribution or recovery of marker enzyme activities. Triplicate 450-μl aliquots were then transferred to polystyrene test tubes and preincubated at 25°C. After 15 min, 50-μl samples were removed and suspended in 950 μl of ice-cold 150 mM NaCl. Immediately thereafter, the remainder of each aliquot (400 μl) was treated with an equivalent volume of bromelain (8 mg or 32 units per ml of sodium phosphate buffer; Sigma Chemical Co., St. Louis, MO) yielding a 1 : 1 (w/w) ratio of enzyme/brush border protein. At selected intervals, 100-μl samples were removed and suspended in 900 μl of ice-cold physiological saline. All samples were centrifuged at $19000 \times g$ for 20 min (4°C). The resultant supernatant fractions and reference samples were analyzed for a series of marker enzyme activities. The pelleted membranes from samples taken after 60 min of proteolysis were solubilized in 1 ml of 0.1% (v/v) Triton X-100, and then diluted 100-fold or more prior to analyses for the same marker enzyme activities. Under these conditions the nonionic detergent had no direct effect on the hydrolase activities monitored.

Treatment with filipin in vitro. Brush borders isolated from six vitamin D-deficient chicks were pooled after sedimentation through sucrose density gradients, and harvested by additional centrifugation [9]. 2-ml aliquots of the resuspended brush borders (4 mg protein/ml of sodium phosphate buffer) were then treated in one of the following ways. In order to determine the effect of the polyene antibiotic on the proteolytic release of hydrolase activities, brush borders were preincubated for 5 min (25°C) and then exposed to filipin (Upjohn Co., Kalamazoo, MI) at a concentration of 5 μg/ml of brush border suspension, or an equivalent volume of vehicle (methanol/water, 1 : 1, v/v) [8], for an additional 15 min. Triplicate aliquots of the filipin- and vehicle-treated suspensions were then removed for limited proteolysis studies, as described above (2 mg/ml each of brush border protein and bromelain, and 2.5 μg/ml filipin, final concentrations).

Alternatively, the effect of a range of filipin concentrations on membrane-bound brush border hydrolase activities was investigated. For this purpose, 2-ml aliquots of the brush border suspension were preincubated for 5 min (25°C) and then exposed to filipin (2.5–10 μg/ml, final concentrations) or an equivalent volume of methanol/water (0.05% methanol, final concentration). After an additional 15-min incubation, samples were removed for analyses of hydrolase activities.

Enzyme and protein analyses. Alkaline phosphatase activity (EC 3.1.3.1) was determined using 5 mM *p*-nitrophenyl phosphate (Sigma) as substrate in 50 mM glycine-NaOH buffer (pH 10), containing 5 mM $MgCl_2$ and 1 mM $ZnCl_2$, final concentrations [10], in a total volume of 0.6 ml. The reaction was terminated by addition of 2.5 ml of 0.02 M NaOH, and product formation assessed spectrophotometrically against standards of *p*-nitrophenol (Sigma). Leucine aminopeptidase activity (EC 3.4.1.3) was determined according to the method of Roncari and Zuber [11] using L-leucine-*p*-nitroanilide (Sigma) as substrate, with the excep-

tion that the reaction was stopped after 30 min of incubation (37°C) with 2.1 ml of 0.02 M NaOH, and product formation analyzed by comparison to standards of *p*-nitroaniline (Sigma). Maltase (EC 3.2.1.20) and sucrase (EC 3.2.1.26) activities were determined by the method of Dahlquist [12] supplemented with Sigma Technical Bulletin No. 510, using maltose and sucrose (Sigma) as substrates, respectively. Protein was analyzed by the method of Lowry et al. [13] using purified bovine serum albumin (Sigma) as standard.

Statistical significance was assessed by the Student's *t*-test for paired sample means.

Results

Vitamin D status and the release of brush border hydrolase activities by bromelain

Bromelain treatment of brush borders isolated from vitamin D-3-treated and deficient chicks revealed significant differences in the solubilized levels of all four hydrolase activities studied (Fig. 1). The data are expressed as the percent of the appropriate enzyme activity in the corresponding reference sample, in order to normalize for vitamin D-mediated alteration in specific activities of the hydrolases [1] (also see below). Thus, al-

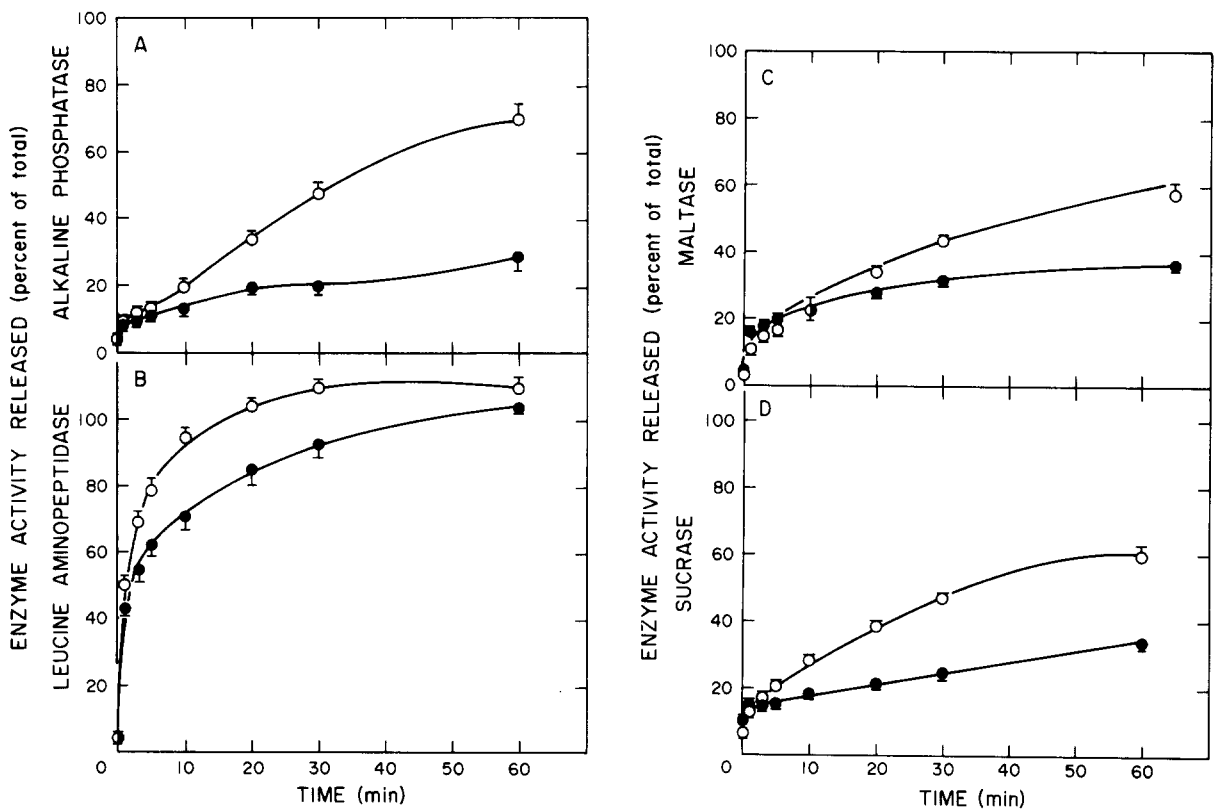

Fig. 1. Release of four brush border hydrolase activities by bromelain. Brush borders isolated from chicks treated with 32.5 nmol of vitamin D-3 96, 72, 48, and 24 h prior to sacrifice (●———●) or vehicle (○———○), were resuspended to 8 mg protein per ml of 50 mM sodium phosphate buffer, pH 6.0, and triplicate aliquots preincubated for 15 min at 25°C. After removal of a zero-time sample (0.4 mg protein), bromelain was added so that final concentrations of brush border protein and protease (6.4 units) were each 4 mg/ml. The final volume of the incubation mixtures was 800 μl. Samples taken at indicated times were diluted with ice-cold 150 mM NaCl and centrifuged at 19000 × *g* for 20 min. Hydrolase activities in the resultant supernatant fractions were related to appropriate levels in corresponding reference samples that had been held on ice to yield percent of total activity released. Values presented represent the mean of triplicate incubations for three independent experiments. The bars in this and subsequent figures represent S.E.

kaline phosphatase levels in supernatant fractions of samples taken after 20 min of proteolysis were found to be significantly less for brush borders isolated from vitamin D-3-treated birds, relative to paired preparations from rachitic chicks ($P < 0.01$; Fig. 1A). A maximal difference in released alkaline phosphatase levels was observed by 30 min, at which time hydrolase activity solubilized from brush borders of vitamin D-replete chicks was 42% of controls ($P < 0.001$). At the end of the 60-min incubation period, soluble alkaline phosphatase levels were found to be 30% and 71% of the activity in corresponding reference samples of brush borders prepared from vitamin D-3-treated and deficient birds, respectively ($P < 0.001$; Fig. 1A).

Within the same series of experiments, the proteolytic release of three additional hydrolase activities was likewise revealed to be lower in brush borders isolated from vitamin D-3-treated chicks (Fig. 1B–1D). Leucine aminopeptidase activity solubilized from brush borders obtained from vitamin D-replete birds was found to be 75% to 80% of corresponding control levels in samples removed 3- to 30 min after addition of bromelain ($P < 0.01$ to < 0.001; Fig. 1B). In samples taken 20 min after the start of the incubation protocol, maltase activity was observed to be less readily released from brush border preparations of vitamin D-3-treated chicks ($P \simeq 0.02$), and by 60 min, soluble disaccharidase activity was 64% of control levels ($P < 0.001$; Fig. 1C). A highly significant, vitamin D-mediated difference in cleavage of sucrase activity was first observed in supernatant fractions of samples taken 10 min after addition of bromelain and at subsequent time points (all, $P < 0.001$). At the end of the incubation period, sucrase activity liberated from brush border preparations of vitamin D-3-treated chicks was 56% of control levels (Fig. 1D).

Vitamin D-mediated differences in recovery of hydrolase activities after proteolysis

Throughout the series of experiments on release of hydrolase activities described above, membrane pellet fractions obtained from samples removed at 60 min of incubation were routinely analyzed for residual marker enzyme activities. Summation of the values determined for the pellet fractions with appropriate hydrolase levels in the corresponding supernatant fractions, indicated that bromelain had selectively degraded certain hydrolase activities. The data presented in Table I demonstrate complete recovery of alkaline phosphatase activity after proteolysis of brush borders isolated from rachitic chicks, but a loss of nearly one half of this activity after exposure of the preparation from vitamin D-3-treated birds to bromelain ($P < 0.01$). No loss of leucine aminopeptidase activity occurred after digestion, whereas approx. 10% of total maltase activity was lost from brush borders obtained from vitamin D-3-deficient, as well as vitamin D-3-treated chicks (Table I). Incomplete recovery of sucrase activity was observed for protease-treated brush border preparations from vitamin D-3-deficient and replete chicks (Table I). However, after exposure to bromelain, approximately twice as much disaccharidase activity was lost from preparations from vitamin D-deficient chicks as from vitamin D-deficient chicks as from vitamin D-treated birds ($P < 0.02$; Table I).

One possible explanation for the substantial inactivation of alkaline phosphatase activity in brush border preparations of vitamin D-3-treated chicks (Table I), is an altered insertion of this hydrolase in the membrane [1] as a function of vitamin D status such that another cleavage site becomes accessible to the action of bromelain. If so, disruption of membrane lipids to increase the accessibility of the entire marker enzyme to proteolysis would result in equivalent losses of alkaline phosphatase activity in brush borders isolated from both vitamin D-3-deficient and vitamin D-3-treated birds. In an attempt to test this hypothesis, aliquots of each brush border preparation were solubilized in the presence of 0.1% (v/v) Triton X-100 and reference samples removed for later analysis. Protease was then added such that final concentrations per ml were 4 mg bromelain and 2 mg brush border protein, and 0.05% (v/v) Triton X-100. After a 60-min incubation (25°C), the total recovery of alkaline phosphatase activity was found to be 99.4 ± 1.4 and 95.1 ± 4.1% for brush border preparations from vitamin D-3-deficient and vitamin D-3-treated chicks, respectively. Inability to detect inactivation of the hydrolase in brush borders prepared from vitamin D-3-treated birds indicates that bromelain was inhibited by

TABLE I

RECOVERY AND DISTRIBUTION OF BRUSH BORDER HYDROLASE ACTIVITIES

Procedures were as described in Fig. 1 for brush border preparations from vitamin D-3-deficient (−D) and treated (+D) chicks. Values presented represent the mean percent of total hydrolase activity in samples taken at 60 min. Numbers in parentheses represent mean specific activities (nmol substrate degraded per min per mg protein) of marker enzymes in reference samples. Mean ± S.E. for three independent experiments.

Enzyme activity	−D			+D		
	Fraction		Recovery	Fraction		Recovery
	Supernatant	Pellet		Supernatant	Pellet	
Alkaline phosphatase	70.6 ± 4.7	28.9 ± 3.3	99.5 ± 7.4 (8800 ± 1900)	29.5 ± 4.6 [a]	27.6 ± 3.8	57.1 ± 7.7 [b] (27400 ± 9850)
Leucine amino-peptidase	110.0 ± 3.0	3.7 ± 0.8	113.7 (905 ± 61)	104.0 ± 1.5	9.9 ± 2.6	113.9 (1080 ± 69)
Maltase	57.9 ± 3.2	29.4 ± 1.5	87.3 (3080 ± 160)	36.9 ± 1.7 [a]	52.0 ± 5.9	88.9 (2140 ± 260)
Sucrase	60.2 ± 3.1	19.7 ± 1.2	79.9 ± 4.1 (552 ± 28)	34.0 ± 2.1 [a]	57.9 ± 1.8	91.9 ± 2.3 [c] (266 ± 28)

[a] $P < 0.001$, relative to corresponding levels in equivalent fractions from vitamin D-deficient chicks.
[b] $P < 0.01$, relative to recovery of activity from brush border preparations of vitamin D-deficient chicks.
[c] $P < 0.02$, relative to recovery of activity from brush border preparations of vitamin D-deficient chicks.

even low concentrations (0.05%) of the nonionic detergent.

Effect of filipin on the proteolytic release of brush border hydrolases

The polyene antibiotic filipin is known to bind membrane cholesterol [8], and mimic under in vitro conditions, the effect of dietary vitamin D-mediated elevation in calcium transport across the intestine [7]. It was of interest therefore to determine whether a membrane-perturbing agent such as filipin could effect alterations in brush border protein topography, as judged by limited proteolysis.

Parallel brush border preparations from six vitamin D-deficient chicks were pooled and aliquots exposed to filipin (5 μg/ml, final concentration) or an equivalent volume of vehicle (methanol/water, 1 : 1, v/v) for 15 min (25°C) prior to the addition of bromelain. A time-course study on release of hydrolase activities was then conducted and the data presented in Fig. 2. When solubilization is expressed as the percent of appropriate hydrolase activity in corresponding reference samples, pretreatment of brush borders with filipin resulted in greatly enhanced release of the four maker enzyme activities, relative to levels from vehicle controls. (Fig. 2). Thus, at 60 min, release of alkaline phosphatase, leucine aminopeptidase, maltase, and sucrase activities from filipin-treated brush borders were 152%, 118%, 138%, and 129%, respectively, of corresponding control levels (Fig. 2A–2D). However, determination of residual hydrolase activities in the membrane pellets after resuspension in the presence of Triton X-100 (see above), indicated that treatment of brush borders with filipin resulted in up to 140% recovery of the marker enzyme activities (data not shown). The data presented in Fig. 3 suggest that such excessive recoveries were not due to direct activation of the soluble hydrolase activities by the antibiotic. A comparison of solubilized alkaline phosphatase, leucine aminopeptidase, maltase, and sucrase specific activities reveals that at 60 min of incubation, enzyme levels from filipin-treated brush borders were 107%, 110%, 92%

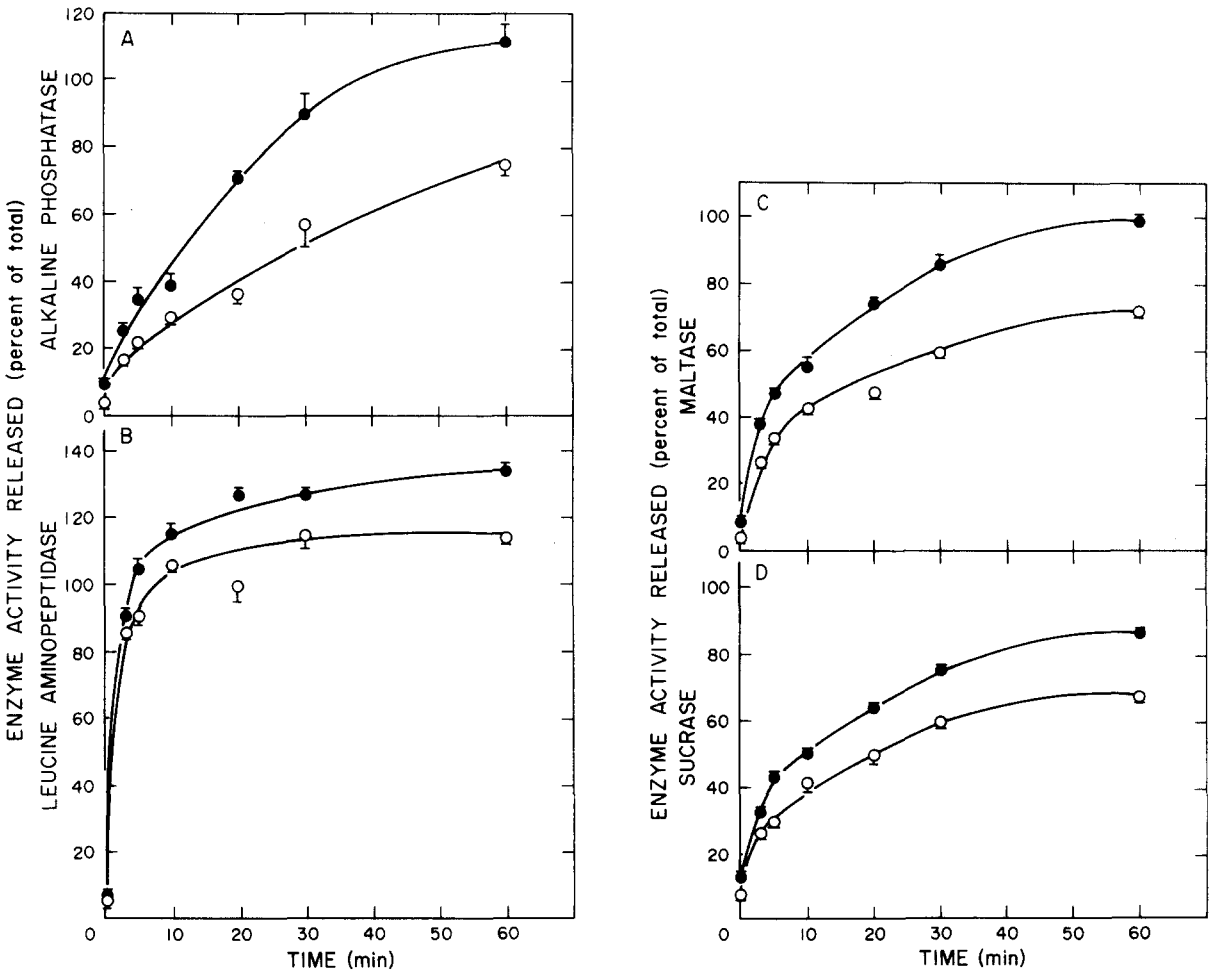

Fig. 2. Effect of filipin treatment in vitro on proteolytic release of hydrolase activities from vitamin D-deficient chicks. Procedures were as described in Fig. 1, except that brush borders were preincubated for 5 min (25°C) before exposure to filipin (5 μg/4 mg brush border protein, ●———●) or an equivalent volume of vehicle (methanol/water, 1 : 1, v/v; ○———○). After an additional 15 min incubation, bromelain was added so that final concentrations of brush border protein and protease were each 2 mg/ml. The final incubation volume was 800 μl. Values presented represent the mean of triplicate incubations.

and 106% of vehicle controls, respectively (Fig. 3A–3D). Moreover, summation of hydrolase specific activities in the supernatant fractions with the appropriate levels in corresponding pellet fractions, indicated filipin pretreatment did not promote selective degradation of any of the four marker enzyme activities tested (data not shown).

However, comparison of specific activities in reference samples revealed a striking effect of filipin on membrane-bound hydrolase activities (Table II, Expt. No. 1). At a final concentration of 5 μg/ml, inhibition of all four hydrolase activities was observed, relative to vehicle control levels, with the disaccharidase activities exhibiting the most sensitive response. In a subsequent experiment, brush borders prepared from vitamin D-deficient chicks were treated with a range of filipin concentrations or an equivalent volume of vehicle. At the lowest concentration tested (2.5 μg/ml), little or no inhibition of hydrolase activities was observed, whereas the next higher concentration (5 μg/ml) again resulted in a pronounced decrease in

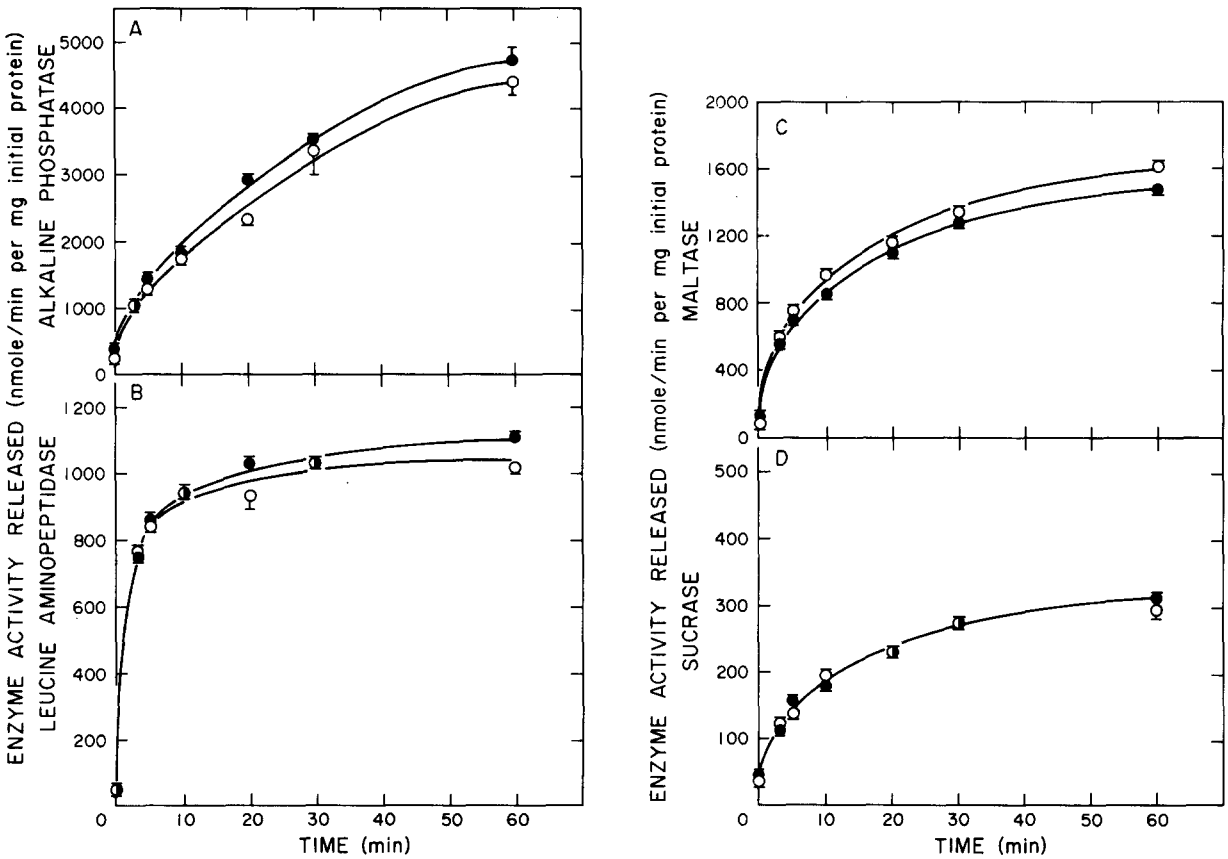

Fig. 3. Effect of filipin treatment in vitro on release of hydrolase activities from brush border preparations of vitamin D-deficient chicks. The data are taken from the experiment presented in Fig. 2 and expressed as the mean specific activities solubilized by proteolysis of brush borders treated with filipin (●———●) or an equivalent volume of vehicle (○———○).

TABLE II

EFFECT OF FILIPIN ON THE SPECIFIC ACTIVITIES OF FOUR MARKER ENZYMES

Brush borders were isolated from vitamin D-deficient chicks and treated with filipin in vitro at the final concentrations indicated, or an equivalent volume of vehicle, as described in Fig. 2. Values are presented for two independent experiments, and represent the mean of triplicate determinations. The activities are expressed as nmol substrate degraded per min per mg protein.

Treatment	Exp. No.	Enzyme activity			
		Alkaline phosphatase	Leucine aminopeptidase	Maltase	Sucrase
Control	1	5970	893	2250	460
Filipin, 5 μg/ml		4200	818	1490	361
Control	2	5000	757	2130	444
Filipin, 2.5 μg/ml		4570	753	2050	405
Filipin, 5 μg/ml		4630	741	1630	334
Filipin, 10 μg/ml		6500	778	2010	397

membrane-bound maltase and sucrase activities (Table II, Expt. No. 2). Of particular interest is the finding that maltase activity in brush borders treated with 5 μg/ml filipin was on the average 71% of levels in vehicle controls, a reduction comparable to that mediated by dietary vitamin D-3 treatment, i.e., 69% of the disaccharidase activity determined in brush borders isolated from rachitic chicks (see Table I). Addition of filipin to 10 μg/ml indicated a biphasic effect of the antibiotic, as hydrolase activities approached or returned to control levels (Table II).

Discussion

The results presented in this communication suggest that limited proteolysis is a useful technique for assessing vitamin D-mediated modifications of intestinal brush border topography, and indicate that vitamin D has pleiotropic effects on intestinal epithelial cells. In a previous investigation, brush borders that were isolated from vitamin D-deficient and treated chicks and subsequently exposed to papain, revealed no difference in alkaline phosphatase release, a greater release of leucine aminopeptidase and maltase in preparations from vitamin D-treated birds, and more efficient solubilization of sucrase in preparations from rachitic chicks [1]. Moreover, no loss of catalytic activity occurred after exposure of brush borders to papain, as judged by complete recovery of all four marker hydrolases in preparations from vitamin D-treated as well as deficient birds.

In contrast, the present work demonstrates that treatment of brush border preparations with bromelain results in lower soluble levels of all four marker enzymes from brush borders isolated from vitamin D-3-replete birds, relative to deficient controls. The dissimilarity between observations in the present and previous study, i.e., relative effects of vitamin D status on the extent of hydrolase release must clearly arise from the different substrate specificities of the two proteases used. The most striking example of such selectivity in cleavage sites is the ineffectiveness of papain in solubilizing alkaline phosphatase from brush borders regardless of vitamin D status [1], whereas bromelain readily increased the marker enzyme activity in supernatant fractions of samples taken from brush border suspensions prepared from rachitic chicks.

The selective inactivation of alkaline phosphatase by bromelain in brush border preparations of vitamin D-3-treated chicks, but complete recovery of the marker hydrolase in paired incubations of brush borders from rachitic birds, could be attributed to a number of causes. Loss of activity could arise from degradation of a vitamin D-induced isoenzyme of alkaline phosphatase, or an activator of the marker hydrolase [14,15]. Alternatively, a vitamin D-dependent modification in the insertion of the brush border enzyme in the lipid matrix could expose a critical amino acid sequence to cleavage by bromelain. However, treatment of isolated brush borders with the membrane-perturbing agent filipin, failed to alter recovery of hydrolase specific activities after proteolysis, relative to similarly digested vehicle controls. This observation does not rule out more subtle changes in lipid composition [16,17] or other potential topographical modifications [1] as explanations for vitamin D-dependent susceptibility of alkaline phosphatase to inactivation by bromelain. Clearly, the combined considerations also apply to the differential recovery of sucrase as a result of proteolysis, reported in the present work.

As noted above, analyses of brush border preparations of vitamin D-deficient chicks treated with filipin or vehicle in vitro, and subsequently exposed to bromelain, indicated that recovery of hydrolase specific activities was unaffected by the presence of the polyene antibiotic. Nor did filipin greatly alter the specific activities of bromelain-released marker hydrolases. Filipin treatment did, however, have a pronounced inhibitory effect on the membrane-bound specific activities of maltase and sucrase, and to a much smaller extent, alkaline phosphatase and leucine aminopeptidase. Further investigation revealed a biphasic response to increasing filipin concentrations. Using a mean cholesterol content of 100 μg per mg brush border membrane protein [9], it can be calculated that a ratio of cholesterol : filipin (w/w) of 8 : 1 inhibited disaccharidase specific activity in a reproducible manner, whereas ratios of 16 : 1 and 4 : 1 produced little effect. These data suggest that inhibition might occur as a result of lipid-matrix constraints [18] arising from sequestered cholesterol, and that addition of a critical concentration of filipin dis-

rupts the membrane bilayer to the extent that the marker enzymes are released from inhibition. Some support for this concept is provided by excess percent recovery of activities in Triton-solubilized pellet fractions of samples prepared after exposure of filipin-treated brush borders to bromelain. The presence of Triton X-100 is known to disrupt complexes of filipin and cholesterol [8], and thus would be expected to abolish inhibitory effects on marker hydrolase activities, so that summation with appropriate supernatant activities would yield much greater than 100% recovery of levels in corresponding reference samples.

For many years filipin has been recognized as a valuable probe for investigating the mechanism of vitamin D-mediated transport of calcium across the intestine [3,19,20]. The present work demonstrates the usefulness of the polyene antibiotic/brush border system as a tool for studying other membrane-related phenomena. For example, filipin effected a reduction in maltase activity comparable to that observed in brush borders isolated from vitamin D-treated birds [1]. Perhaps of greater significance is the observation that the inhibitory effect of filipin was more apparent for two of the four marker hydrolase specific activities monitored. The relative absence of an effect on the specific activities of alkaline phosphatase and leucine aminopeptidase suggests that these enzymes are not randomly distributed [21,22], but are localized in a micro-domain of brush border lipids, that is distinct from the environment of the disaccharidases. The existence of discrete 'frozen lipid domains' has been noted in other cell types [23], and their potential existence in brush border membranes of intestinal epithelial cells may have a bearing upon vitamin D-mediated calcium transport in the intestine.

Acknowledgments

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