ELSEVIER

Biochimica et Biophysica Acta 1190 (1994) 329–336

BBA Biochimica et Biophysica Acta

Regulation of calmodulin binding to the ATP extractable 110 kDa protein (myosin I) from chicken duodenal brush border by 1,25-$(OH)_2D_3$

Reinhard Kaune *, Scott Munson, Daniel D. Bikle

Veterans Administration Medical Center, and University of California, San Francisco, CA 94121, USA

(Received 18 May 1993; revised manuscript received 3 December 1993)

Abstract

In earlier studies we observed that the active vitamin D metabolite 1,25-$(OH)_2D_3$ increased the calmodulin content of purified duodenal brush-border membrane vesicles where it bound principally to the 110 kDa protein myosin I. In this study we further evaluated the regulation of calmodulin binding to ATP releasable myosin I. Whole brush borders (BB) or purified brush-border membrane vesicles (BBMV) were prepared from duodena of vitamin D-deficient rachitic chicks treated 12–18 h before killing with either 625 pmol 1,25-$(OH)_2D_3$ or vehicle. The ATP extractable myosin I from BB resulted in an 1.6-fold increase of calmodulin binding to the 110 kDa band after treatment with 1,25-$(OH)_2D_3$. In contrast to BB, ATP extraction of myosin I from purified BBMV required alamethicin for ATP entry. As for BB extracts, calmodulin binding to the 110 kDa band in BBMV extracts was also increased about 2.4-fold by 1,25-$(OH)_2D_3$. It was concluded that both intact BB and purified BBMV showed the same type of increase in calmodulin binding to ATP releasable myosin I by 1,25-$(OH)_2D_3$. To see whether 1,25-$(OH)_2D_3$ increased the intrinsic affinity of calmodulin binding to myosin I, the ATP extractable myosin I from BB was purified from rachitic chicks treated with 1,25-$(OH)_2D_3$ or vehicle. In contrast to ATP extracts of BB or BBMV, calmodulin binding to the purified myosin I was not different between preparations from 1,25-$(OH)_2D_3$- or vehicle-treated chicks. We conclude that 1,25-$(OH)_2D_3$ does not change the affinity of calmodulin binding to myosin I but increases the amount of myosin I in the membrane or alters its ATP releasability. It was further investigated whether phosphorylation is involved in these 1,25-$(OH)_2D_3$ dependent posttranslational changes of myosin I. Phosphorylation of brush-border membrane proteins in vivo was performed by incubation of [^{32}P]P_i in the lumen of a ligated duodenal loop in situ for 15 min. Brush-border membrane proteins were phosphorylated in vitro by incubating BB or BBMV with [γ-^{32}P]ATP for 1 min. Incubation experiments in vivo and in vitro in fact resulted in phosphorylation of several proteins including 110 kDa proteins. However, there was no specific effect of 1,25-$(OH)_2D_3$ on phosphorylation of 110 kDa proteins. We conclude that the effects of 1,25-$(OH)_2D_3$ on protein phosphorylation are minimal and not likely to explain 1,25-$(OH)_2D_3$ stimulated calmodulin binding to ATP extractable brush-border membrane myosin I and 1,25-$(OH)_2D_3$ stimulated changes of calcium uptake across the brush-border membrane.

Key words: Brush-border membrane vesicle; Calcium ion transport; 1,25-Dihydroxyvitamin D_3; Myosin I

1. Introduction

It is well known that the active duodenal absorption of Ca^{2+} occurs under the control of 1,25-$(OH)_2D_3$, the active metabolite of vitamin D_3 [1–3]. The mechanism by which 1,25-$(OH)_2D_3$ increases Ca^{2+} absorption is still uncertain. The best known effect of 1,25-$(OH)_2D_3$ on enterocytes is induction of calbindin-D synthesis, an intracellular Ca^{2+} binding protein which may facilitate calcium movement through the cytoplasm [4]. A second effect is increased Ca^{2+} movement across the brush-border membrane as measured with purified brush-border membrane vesicles [5–7]. This appears to be

* Corresponding author. Permanent address: Department of Physiology, School of Veterinary Medicine, Bischofsholer Damm 15, D-3000 Hannover 1, Germany. Fax: +49 511 8567687.

Abbreviations: BBMV, purified brush-border membrane vesicle(s); BB, whole brush borders; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 25-OHD_3, 25-hydroxyvitamin D_3; AP, activity of alkaline phosphatase; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; DFP, diisopropyl fluorophosphate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; CPM, counts per min.

SSDI 0005-2736(93)E0385-N

mediated by another calcium binding protein, calmodulin. The increased Ca^{2+} uptake into brush-border membrane vesicles following 1,25-$(OH)_2D_3$ administration is accompanied by increased calmodulin binding to a 110 kDa calmodulin binding protein (myosin I) and can be inhibited with calmodulin antagonists like trifluoperazine [8,9]. Furthermore, when membrane vesicles were prepared from different cells along the villus-crypt axis, again Ca^{2+} uptake, calmodulin content and binding to myosin I were correlated [10].

Myosin I appears to link the membrane to the actin core of the microvillus [11]. It binds calmodulin even in the presence of EGTA (i.e., absence of Ca^{2+}) and binds to actin in the absence but not in the presence of ATP [12]. It has ATPase activity which can be stimulated by K^+ in the absence of divalent ions (K-EDTA-ATPase) or by Ca^{2+} and K^+ and to a smaller amount by Mg^{2+} and F-actin [13]. This pattern is typical for myosins, the family of proteins to which brush-border myosin I is now known to belong on the basis of its primary sequence [14]. Although myosin I has mechanoenzyme activity, the function of this protein in vivo, and the physiological meaning of the calmodulin binding is not known; we postulate that it may be involved in the mechanism by which 1,25-$(OH)_2D_3$ increases Ca^{2+} uptake across the brush-border membrane.

We therefore investigated whether 1,25-$(OH)_2D_3$ stimulates the affinity of calmodulin binding to brush-border myosin I by purifying this protein from vitamin D-deficient and 1,25-$(OH)_2D_3$ treated chicks and determining calmodulin binding to the purified protein.

Unlike the induction of calbindin synthesis, the increased binding of calmodulin to the membrane bound myosin I occurs at the same time as increased calcium uptake across the brush-border membrane [5] and is not blocked by cycloheximide [15]. Thus the changes in myosin I and its binding to calmodulin presumably reflect posttranslational modifications. Phosphorylation of membrane proteins is one way to regulate membrane functions, as is the case in other calcium transport membranes [16,17] or membrane bound proteins [18]. Thus we evaluated the ability of 1,25-$(OH)_2D_3$ to alter brush-border membrane protein phosphorylation in vivo by using the ligated duodenal loop preparation, and in vitro using purified whole brush borders or purified brush-border membrane vesicles. The effects of 1,25-$(OH)_2D_3$ on brush-border membrane protein phosphorylation were compared to the effects of 1,25-$(OH)_2D_3$ on calcium transfer, alkaline phosphatase activity in the brush-border membrane and calmodulin binding to myosin I.

2. Materials and methods

1-day-old cockerels were raised for 3–4 weeks on a vitamin D-deficient diet (Teklad, Madison Wi 53711, diet no. 75007 containing 0.8% calcium and 0.6% phosphorus). The 1,25-$(OH)_2D_3$ or vehicle was given orally in doses of 625 pmol in 100 μl propylene glycol 12–18 h before killing. Calcium transport in vivo was measured by the in situ duodenal loop technique [19] and serum calcium concentration by atomic absorption spectrometry.

Whole brush borders (BB) of chick duodenum were isolated as described by Swanljung et al. [20] in a modified way. The chicks were killed by decapitation using a guillotine. The duodenum was quickly removed, rinsed with ice-cold saline, and placed on ice in a buffer (10 mmol/l KH_2PO_4, 150 mmol/l NaCl (pH 7.5)) containing dithiothreitol (DTT, 1 mmol/l), phenylmethylsulfonyl fluoride (PMSF, 0.2 mmol/l), NaN_3 (0.02%), and the proteinase inhibitors aprotinin (5 μg/ml), leupeptin (1 μg/ml), pepstatin A (5 μg/ml) and diisopropyl fluorophosphate (DFP, 1 mmol/l). All future steps were carried out on ice or in a cold room at 4°C. The tissues were transferred into a beaker with 76 mmol/l Na_2PO_4, 19 mmol/l KH_2PO_4, 12 mmol/l EDTA (pH 7.0) containing DTT, PMSF, NaN_3, and the proteinase inhibitors as described above and stirred with a magnetic stir bar. After 30 min of stirring, the residual epithelial and muscle layers were removed with a forceps and the suspended cells pelleted at $250 \times g$ for 10 min in a refrigerated high speed centrifuge. Cells were washed once and suspended in the homogenization buffer (10 mmol/l imidazole chloride, 4 mmol/l EDTA, 1 mmol/l EGTA (pH 7.3) with DTT, PMSF, NaN_3, and the proteinase inhibitors as described above) and homogenized 3×5 s with a Brinkmann polytron (Westbury, NY, setting 2). The homogenate was centrifuged at $800 \times g$ for 10 min and washed twice in the homogenization buffer and twice in a wash buffer (10 mmol/l imidazole chloride, 75 mmol/l KCl, 5 mmol/l $MgCl_2$, 1 mmol/l EGTA (pH 7.3), with DTT, PMSF, NaN_3, and the proteinase inhibitors as described above without DFP). The final pellet contains brush borders and nuclei. The nuclei were removed by resuspending the pellet in 50% sucrose in wash buffer, overlaid with 40% sucrose in wash buffer, and centrifuged at $100\,000 \times g$ for 90 min. The brush borders migrated to the interface [21]. They were removed with a syringe, diluted 10 times with wash buffer, pelleted at $10\,000 \times g$ for 10 min, and washed once again with wash buffer.

Brush-border membrane vesicles (BBMV) were prepared using the method of Max et al. [22] with 0.1 mmol/l PMSF in all buffers and three 10 s bursts of a Brinkmann Polytron instead of a Waring blender.

ATP extracts of the brush borders or brush-border membrane vesicles were obtained by suspending in ATP extraction buffer (10 mmol/l imidazole chloride, 0.2 M KCl, 5 mmol/l $MgCl_2$, 5 mmol/l ATP, 1 mmol/l EGTA (pH 6.8), with DTT and PMSF as described

above) and subsequent centrifugation with 100 000 × *g* for 30 min. For the extraction of brush-border membrane vesicles 0.2% alamethicin was added to the extraction buffer, to facilitate the entry of ATP into the vesicles.

For the purification of myosin I a crude brush-border preparation of 25–30 duodena was used as described above without removing the nuclei by a sucrose gradient. An ATP extract of this preparation was run over a sepharose Cl-4B column (5 × 90 cm, Pharmacia) and two FPLC columns, Mono Q and Mono S (both type HR 5/5, Pharmacia) following the method described

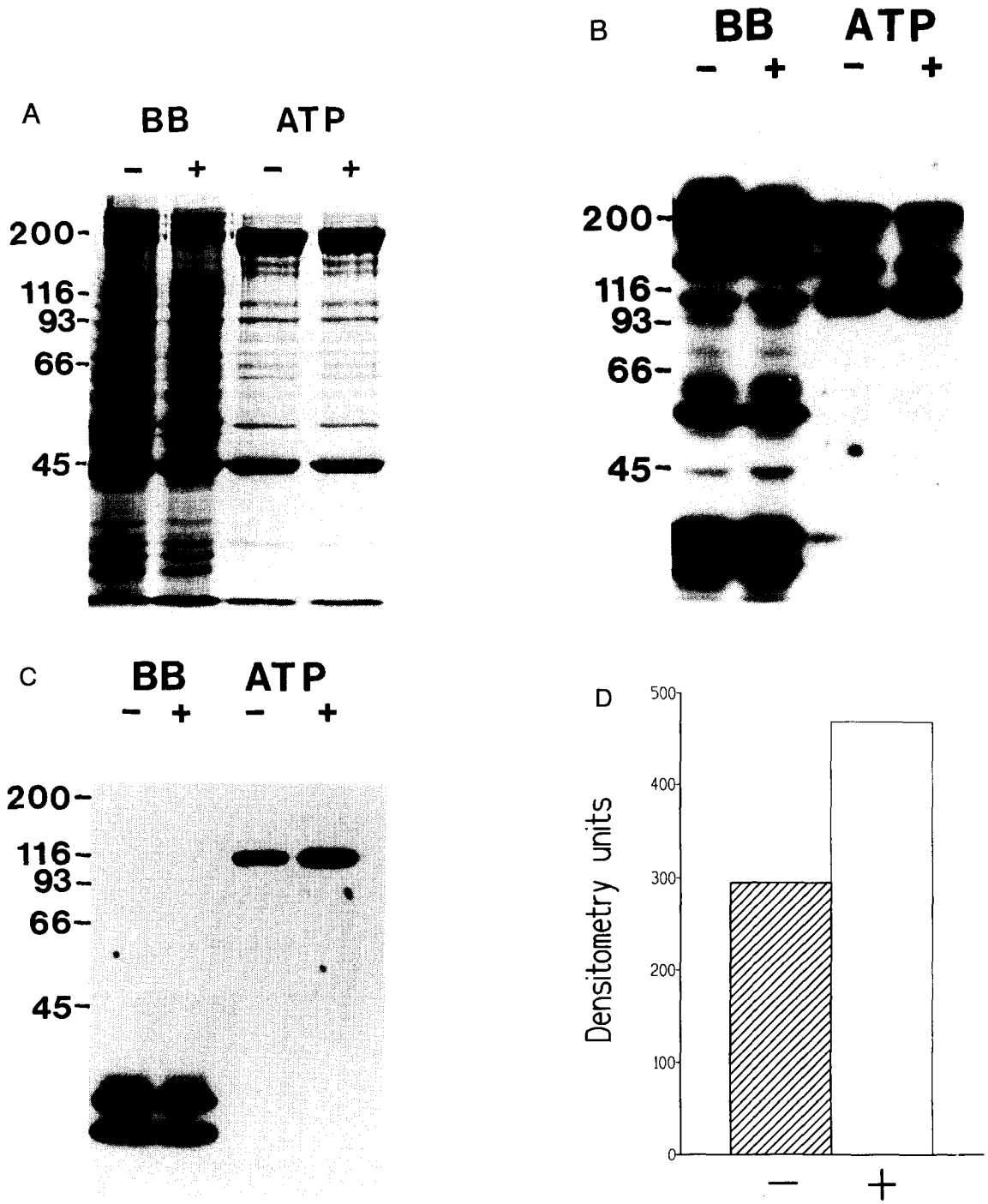


Fig. 1. SDS-PAGE and calmodulin overlay of whole brush borders (BB) and ATP extracts (ATP) prepared from vitamin D-deficient chicks treated with 1,25-$(OH)_2D_3$ (+) or vehicle (−). The positions of the molecular weight markers are shown on the margins. (A) Coomassie-stained gels. (B) Corresponding autoradiogram of calmodulin binding in the presence of 1 mmol/l calcium. (C) Corresponding autoradiogram of calmodulin binding in the presence of 1 mmol/l EGTA. (D) Quantification of the autoradiogram shown in (C, ATP).

by Swanljung et al. [20]. The fractions containing myosin I were identified by K-EDTA-ATPase activity measured with the method of Korn et al. [23].

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out by the method of Laemmli [24] using 8% polyacrylamide gels.

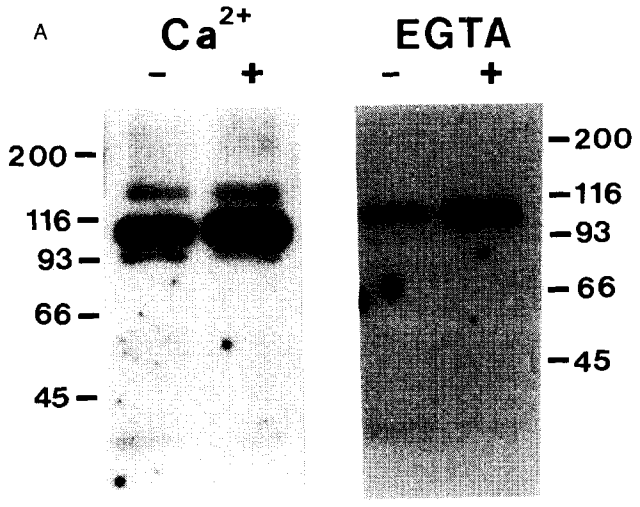
Calmodulin overlay and scanning of Coomassie-stained gels or autoradiograms were performed as described previously [8]. Radioiodination of calmodulin was carried out using the Iodo-Gen iodinating reagent (Pierce), and ^{125}I-calmodulin was separated from unbound ^{125}I with Sephadex G-25 columns.

Phosphorylation of brush-border membrane proteins in vivo: The chicks were anesthetized by pentobarbital. Three-hundred μl of (mmol/l) 120 NaCl, 25 Hepes, 6 KCl, 1 $MgSO_4$, 20 dextrose (pH 7.4), containing 200 μCi of [^{32}P]P_i or [^{33}P]P_i (Amersham, Arlington Heights, IL) were injected into the lumen of the proximal duodenum. The duodenum was ligated at both ends to form a 5-cm-long loop with blood supply intact. After 15 min the chick was killed, the loop was removed, drained of luminal contents, and the mucosa was removed by scraping with glass slides. Brush-border membrane vesicles were then prepared as described above.

Phosphorylation of brush-border membrane proteins in vitro: Phosphorylation in BBMV was done by a modification of the procedure of Miyamoto et al. [26]. 200 μg of vesicle protein was incubated in 200 μl of 50 mmol/l β-glycerophosphate, 10 mmol/l Mg-acetate, 2 mmol/l theophylline, 10 mmol/l NaF (pH 6.5), with 0.2% alamethicin (a gift from J.E. Grady, Upjohn, Kalamazoo, MI). The vesicles were incubated for 5 min at 37°C before the addition of 50 μl of the above buffer containing 5 μCi [γ-^{32}P]ATP (Amersham, Arlington Heights, IL) and $5 \cdot 10^{-10}$ mol ATP. After an additional 1-min incubation, 100 μl ice-cold 15% trichloroacetic acid (TCA) was added. The TCA-precipitable material was washed twice with water. Modifications of this general protocol for specific experiments are described in the text and Figure legends.

Phosphorylation of whole brush-border preparations was carried out by incubating brush-border preparations at room temperature with extraction buffer (10 mmol/l imidazole chloride, 200 mmol/l KCl, 5 mmol/l $MgCl_2$, 1 mmol/l EGTA, 10 μmol/l ATP (pH 6.8), with DTT and PMSF as described above) in the presence of 200 μCi/ml [γ-^{32}P]ATP. After 1 min of incubation, unlabeled ATP was added to a final concentration of 5 mmol/l and the sample centrifuged at 100000 $\times g$ for 30 min at 4°C. The supernatant contained ATP releasable proteins.

Electrophoresis and quantification of protein phosphorylation: The brush borders or brush-border membrane vesicles phosphorylated in vivo or in vitro were subjected to SDS-PAGE with 8% polyacrylamide gels as described above. Immediately after preparation 20 μl aliquots were mixed with 7 μl 4 × SDS-PAGE stop solution (0.25 mol/l Tris, 40% glycerol, 4% SDS, 120 mmol/l DTT (pH 6.8)) and heated to 95°C for 5 min before separation by SDS-PAGE. In most experiments the gels were stained with Coomassie blue, dried, and exposed to X-Omat film (Eastman Kodak Co., Rochester, NY) for 7–24 h at −80°C. The autoradio-



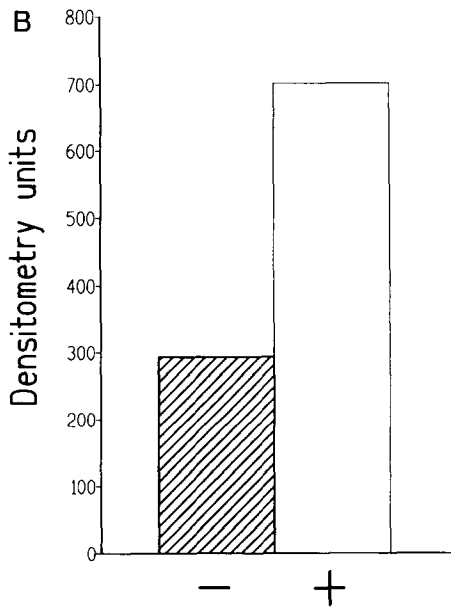


Fig. 2. (A) Calmodulin binding of myosin I of an ATP + alamethicin extract of brush-border membrane vesicles from vitamin D-deficient chicks treated with 1,25-$(OH)_2D_3$ (+) or vehicle (−) in the presence of 1 mmol/l Ca^{2+} or EGTA. (B) Quantification of calmodulin binding in the presence of EGTA.

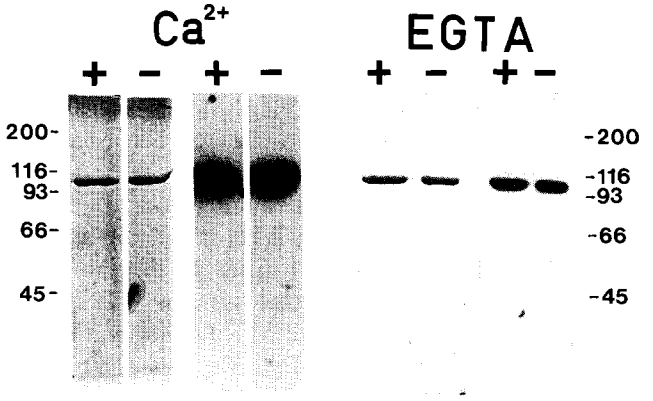


Fig. 3. SDS-PAGE and calmodulin overlay of the purified myosin I from vitamin D-deficient chicks treated with 1,25-$(OH)_2D_3$ (+) or vehicle (−) in the presence of 1 mmol/l Ca^{2+} or EGTA. The positions of the molecular weight markers are shown on the left and right margins. Left two lanes in the presence of Ca^{2+} or EGTA: Coomassie-stained gels; right two lanes: corresponding autoradiograms.

grams were scanned by a densitometer (E-C Apparatus, St. Petersburg, FL) with identical settings for each experiment.

Other methods. Calcium accumulation by brush-border membrane vesicles was determined as previously described [5]. The results are expressed in terms of calcium accumulated during the 15 min incubation period. Alkaline phosphatase activity was determined by the method of Hausamen et al. [27]. Protein determinations were performed by the method of Lowry et al. [28], or a Coomassie blue kit from Bio-Rad (Richmond, CA) using BSA as standard protein. All chemicals, unless otherwise described, were reagent grade and are available from commercial suppliers.

3. Results

The level of vitamin D deficiency in the chicks used for these experiments and their responsiveness to 1,25-$(OH)_2D_3$ were verified by measuring the Ca^{2+} concentration in serum and Ca^{2+} transport in vivo. The serum Ca^{2+} concentration of three weeks old D-deficient chicks was 1.76 ± 0.28 mmol/l ($n = 18$) and could be raised to 2.20 ± 0.22 mmol/l ($n = 20$) by treatment with 1,25-$(OH)_2D_3$ 16 h before. Duodenal Ca^{2+} transport measured as appearance of ^{45}Ca in serum after 15 min of in situ incubation of $^{45}Ca^{2+}$ in ligated duodenal loops was 2113 ± 406 CPM/200 μl ($n = 4$) in the vehicle-treated and 7322 ± 916 CPM/200 μl ($n = 5$) in the 1,25-$(OH)_2D_3$-treated birds.

Fig. 1 shows that calmodulin binding to the ATP extractable myosin I from BB is increased in 1,25-$(OH)_2D_3$-treated animals. This increase was most pronounced in the presence of EGTA. Based on densitometry of the autoradiogram shown in Fig. 1C, an 1.6 fold increase of calmodulin binding to ATP extractable myosin I was seen (Fig. 1D).

In contrast to BB preparations, ATP extraction of purified BBMV resulted in only 1.9% protein solubilized in comparison to about 10% solubilized from BB preparations. However, with the addition of alamethicin (0.2%) to the extraction buffer, 14% of the proteins from BBMV were solubilized. Using alamethicin, the ATP soluble portion of myosin I in vesicles

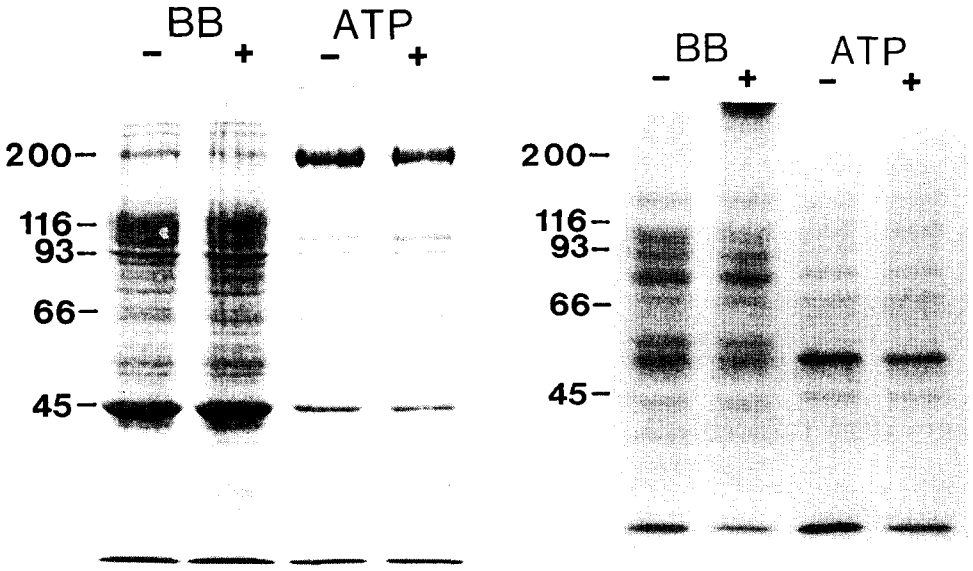


Fig. 4. SDS-PAGE and phosphorylation of brush borders (BB) and ATP extracts (ATP) prepared from vitamin D-deficient chicks treated with 1,25-$(OH)_2D_3$ (+) or vehicle (−). The positions of the molecular weight markers are shown on the left margins. [^{32}P]ATP was added prior to ATP extraction. Left panel: Coomassie-stained gels. Right panel: corresponding autoradiogram.

Table 1
Specific activity, enrichment, and recovery of K-EDTA-ATPase during purification of myosin I

	K-EDTA-ATPase		
	specific activity (μmol/min per mg)	enrichment	recovery (%)
Homogenate	0.0021	–	100
ATP-extract	0.0217	10	51
Gel filtration	0.0252	12	17
Mono Q	0.1298	62	5
Mono S	0.3200	152	1.3

The homogenate was prepared from the duodena of 30 chicks which had been treated with 1,25-$(OH)_2D_3$.

was in the same range as for whole brush borders. Fig. 2 shows that ATP plus alamethicin extracts of brush-border membrane vesicles from 1,25-$(OH)_2D_3$ treated birds also showed an increase in calmodulin binding to the 110 kDa band when compared with that from vehicle-treated birds. The increase shown in Fig. 2A in the presence of EGTA was 2.4-fold (Fig. 2B).

To see whether the intrinsic affinity of myosin I to bind calmodulin is affected by 1,25-$(OH)_2D_3$, ATP solubilized myosin I was purified from duodena of vitamin D-deficient and 1,25-$(OH)_2D_3$-treated animals. The amount obtained from 25–30 birds was 0.4–0.7 mg. The specific activity of K-EDTA-ATPase during purification of myosin I from 1,25-$(OH)_2D_3$-treated birds is shown in Table 1. Neither the specific activity of the enzyme nor the enrichment pattern were strikingly different between 1,25-$(OH)_2D_3$ or vehicle-treated birds.

The purified protein was homogenous by SDS-PAGE except for calmodulin. When the same amount of ATP solubilized myosin I from D deficient and 1,25-$(OH)_2D_3$-treated birds was run on SDS-PAGE gels, there was no difference in calmodulin binding with the overlay technique (Fig. 3). This means that 1,25-$(OH)_2D_3$ would increase the amount of myosin I in the BB or altered its releasibility by ATP without altering its intrinsic affinity for calmodulin.

The next experiments were designed to see, whether 1,25-$(OH)_2D_3$ can increase the membrane binding or ATP releasibility of myosin I through changes in endogenous phosphorylation. Fig. 4 shows a Coomassie-stained gel and the corresponding autoradiogram of an in vitro phosphorylation experiment with whole brush borders. Several proteins including a 110 kDa-protein could be phosphorylated by this way but no major differences following 1,25-$(OH)_2D_3$ treatment were seen.

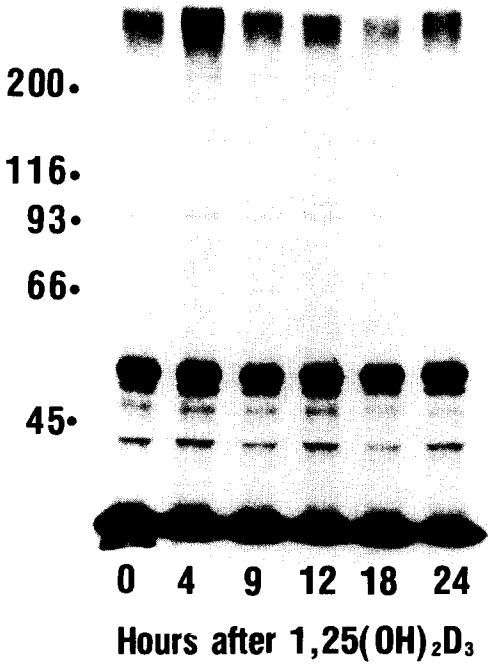


Fig. 5. Effect of 1,25-$(OH)_2D_3$ on the in vitro phosphorylation of proteins in a brush-border membrane vesicle preparation. This is an autoradiogram of a SDS-PAGE gel of 300 μg brush-border membrane protein samples. The samples were prepared from the duodenal mucosa of chicks given 1,25-$(OH)_2D_3$ at 0, 4, 9, 12, 18, and 24 h before preparation of the vesicles. The vesicles were phosphorylated in vitro in the presence of 0.2% alamethicin before SDS-PAGE. The position of the molecular weight markers is shown on the left margin.

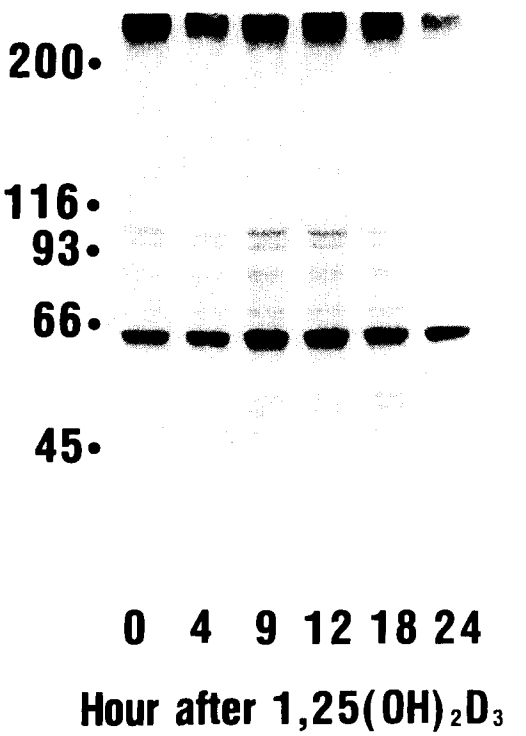


Fig. 6. Effect of 1,25-$(OH)_2D_3$ on the in vivo phosphorylation of brush-border membrane proteins. This is an autoradiogram of a SDS-PAGE gel containing 300 μg protein of brush-border membrane samples. Brush-border membrane vesicles were prepared from the duodenal mucosa of chicks given 1,25-$(OH)_2D_3$ at 0, 4, 9, 12, 18, and 24 h before incubation of the duodenum with ^{32}P in vivo. The position of the molecular weight standards is depicted on the left margin.

Table 2
The effect of 1,25-$(OH)_2D_3$ on Ca^{2+} accumulation and on alkaline phosphatase activity of brush-border membrane vesicles

	Hours after 1,25-$(OH)_2D_3$ administration					
	0	4	9	12	18	24
Ca^{2+} *	7.94 ± 0.11	9.89 ± 0.33	10.85 ± 0.60	11.04 ± 0.30	10.34 ± 0.32	9.96 ± 0.39
AP **	3.23 ± 0.03	3.33 ± 0.06	9.62 ± 0.29	8.42 ± 0.16	7.52 ± 0.01	9.28 ± 0.10

The brush-border membrane vesicles used for this experiment were from the same preparation used for the data in Fig. 5.
* Calcium accumulation in nmol/mg protein ± range of duplicate determinations.
** Alkaline phosphatase activity in nmol/min per μg protein ± range of duplicate determinations.

Fig. 5 shows an autoradiogram of the SDS-PAGE separation of proteins from brush-border membrane vesicles prepared from chicks given 1,25-$(OH)_2D_3$ at indicated time points before the duodena were obtained and incubated for 1 min with [γ-^{32}P]ATP in the presence of 0.2% alamethicin. No consistent changes in the phosphorylation profile were observed. The increased phosphorylation observed in the 4-h sample in Fig. 6 was not seen in other experiments.

Fig. 6 shows an autoradiogram of the SDS-PAGE separation of brush-border membrane vesicle proteins phosphorylated in vivo from chicks given 1,25-$(OH)_2D_3$ at indicated time points before the duodena were obtained. Under these conditions phosphorylation of a 110 kDa protein and three other proteins is increased 9–18 h after 1,25-$(OH)_2D_3$ administration. The 110 kDa protein was maximally phosphorylated 9 h after 1,25-$(OH)_2D_3$ administration, but by 24 h, any increase above the untreated sample (designed as 0 h in the figure) was no longer present. The pattern of phosphorylation depicted in Fig. 6 remained unchanged, regardless of whether $^{32}PO_4^{3-}$ or $^{33}PO_4^{3-}$ was used.

In contrast to the lack of effect of 1,25-$(OH)_2D_3$ on protein phosphorylation both calcium accumulating ability and alkaline phosphatase activity measured in these same preparations were increased by the prior administration of 1,25-$(OH)_2D_3$ (Table 2).

4. Discussion

A primary purpose of this study was to explore the mechanism by which calmodulin binding to brush-border myosin I, which we believe is involved in calcium transport across the brush-border membrane, is increased by 1,25-$(OH)_2D_3$ and whether phosphorylation is involved in this mechanism. Our results show that 1,25-$(OH)_2D_3$ does not increase the calmodulin content of the brush border by increasing its affinity for the ATP extractable myosin I because equal amounts of myosin I purified from vitamin D-deficient and 1,25-$(OH)_2D_3$-treated chicks showed equivalent binding to calmodulin. The fact that calmodulin binding of the purified protein is not different in vitamin D-deficient and 1,25-$(OH)_2D_3$-treated animals is in apparent contrast to former results which show that calmodulin binding to the 110 kDa band of brush-border membrane vesicles is increased after 1,25-$(OH)_2D_3$ treatment [8,10]. However, this conflict may be resolved if in 1,25-$(OH)_2D_3$-treated birds more myosin I stays with the membrane during vesicle preparation or if the membrane bound myosin I is altered by 1,25-$(OH)_2D_3$ with respect to calmodulin binding.

1,25-$(OH)_2D_3$ may increase the amount of myosin I in BBMV by stimulating membrane binding. The amount of 110 kDa protein and the calmodulin binding to the 110 kDa band in an ATP and alamethicin extract of BBMV is twice as great when the BBMV originate from chicks treated with 1,25-$(OH)_2D_3$. When combined with the findings of Howe et al. [25] who found that vitamin D reduced the calmodulin binding to the 110 kDa band of demembranated microvillus cores, these results support the possibility that 1,25-$(OH)_2D_3$ may increase the binding of myosin I to the membrane and therefore the calmodulin binding and content of BBMV.

Further experiments were done to see whether these changes in the behavior of myosin I were achieved by endogenous phosphorylation as shown for other proteins [18]. In our in vivo and in vitro experiments a protein of about 110 kDa is phosphorylated, suggesting that myosin I is a protein kinase substrate. This is consistent with results reported by Howe and Mooseker [29].

Our in vivo experiments suggest a transient increase in phosphorylation of myosin I after 1,25-$(OH)_2D_3$ administration, but the effect was not specific for this protein nor was it observed in vitro using preparations of whole brush borders or purified brush-border membrane vesicles. Therefore it is likely that the increased phosphorylation in vivo is due to increased ^{32}P uptake by the gut from the 1,25-$(OH)_2D_3$-treated animals.

Other studies suggest the involvement of protein

phosphorylation in the nongenomic action of 1,25-$(OH)_2D_3$. De Boland and Norman [30] reported evidence for the involvement of protein kinase C and other kinases in 1,25-$(OH)_2D_3$ mediated stimulation of Ca^{2+} transport. The rapid 1,25-$(OH)_2D_3$ effects reported by these authors were observed during the first hour after 1,25-$(OH)_2D_3$ exposure and were found only in normal vitamin D replete chicks. However, maximal effects of 1,25-$(OH)_2D_3$ on calmodulin binding to the brush border and Ca^{2+} uptake into brush-border membrane vesicles from vitamin D-deficient chicks are seen several hours after 1,25-$(OH)_2D_3$ treatment [5].

In conclusion, the effect of 1,25-$(OH)_2D_3$ on calmodulin binding to proteins of the duodenal brush-border membrane appears to have the following characteristics. 1,25-$(OH)_2D_3$ increases calmodulin binding to BBMV by increasing binding to myosin I. Previous studies have shown that this is not the result of increased protein synthesis [15], nor is it a result of net changes in phosphorylation which might be expected to alter calmodulin binding to myosin I or alter myosin I binding to the brush-border membrane (this paper). ATP releases more myosin I from BBMV prepared from duodena of 1,25-$(OH)_2D_3$-treated rachitic chicks than from BBMV prepared from vehicle-treated controls. However, the purified protein does not differ in its ability to bind calmodulin when taken from 1,25-$(OH)_2D_3$- versus vehicle-treated chicks. Taken together this means that 1,25-$(OH)_2D_3$ increases the amount of myosin I in the brush-border membrane or its releasability by ATP.

The mechanism by which 1,25-$(OH)_2D_3$ increases myosin I and thus calmodulin in the brush-border membrane remains uncertain and experiments to explore this mechanism are in progress.

5. Acknowledgements

Supported by grants from the Veterans Administration and the National Institute of Health (AM28116) and the Deutsche Forschungsgemeinschaft (Ka 740/3-1).

6. References

[1] Van Os, C.H. (1987) Biochim. Biophys. Acta 906, 195–222.
[2] Norman, A.W. (1987) J. Nutr. 117, 797–807.
[3] DeLuca, H.F. (1988) FASEB J. 2, 224–236.
[4] Bronner, F. (1990) Miner. Electrolyte Metab. 16, 94–100.
[5] Bikle, D.D., Munson, S. and Zolock, D.T. (1983) Endocrinology 113, 2072–2080.
[6] Liang, C.T., Barnes, J., Balakir, R.A. and Sacktor, B. (1986) J. Membr. Biol. 90, 145–156.
[7] Kaune, R., Kassianoff, I., Schröder, B. and Harmeyer, J. (1992) Biochim. Biophys. Acta 1109, 187–194.
[8] Bikle, D.D. and Munson, S. (1985) J. Clin. Invest. 76, 2312–2316.
[9] Bikle, D.D., Munson, S. and Chafouleas, J. (1984) FEBS Lett. 174, 30–33.
[10] Bikle, D.D. and Munson, S. (1986) Endocrinology 118, 727–732.
[11] Mooseker, M.S. (1985) Annu. Rev. Cell Biol. 1, 261–293.
[12] Verner, K. and Bretscher, A. (1984) J. Cell Biol. 100, 1455–1465.
[13] Collins, J. and Borysenko, C. (1984) J. Biol. Chem. 259, 14128–14135.
[14] Garcia, A., Coudrier, E., Carboni, J., Anderson, J., Vandekerkhove, J., Mooseker, M., Louvard, D. and Arpin, M. (1989) J. Cell Biol. 109, 2895–2903.
[15] Bikle, D.D., Zolock, D.T., Morrissey, R.L. and Herman, R.H. (1978) J. Biol. Chem. 253, 484–488.
[16] Rinaldi, M.L., Capony, J.P. and Demaille, J.G. (1982) J. Mol. Cell. Cardeol. 14, 279–289.
[17] Weller, M. and Laing, W. (1979) Biochim. Biophys. Acta 551, 406–419.
[18] Thelen, M., Rosen, A., Nairn, A.C. and Aderem, A. (1991) Nature 351, 320–322.
[19] Morrissey, R.L., Zolock, D.T., Bikle, D.D., Empson, R.N. and Bucci, T.J. (1978) Biochim. Biophys. Acta 538, 23–33.
[20] Swanljung-Collins, H., Montibeller, J. and Collins, J.H. (1987) Methods Enzymol. 139, 137–148.
[21] Keller, T.C.S. and Mooseker, M.S. (1982) J. Cell Biol. 95, 943–959.
[22] Max, E.E., Goodman, D.B.P. and Rasmussen, H. (1978) Biochim. Biophys. Acta 511, 224–239.
[23] Korn, E.D., Collins, J.H. and Maruta, H. (1985) Methods Enzymol. 85, 357–363.
[24] Laemmli, U.K. (1970) Nature 227, 680–685.
[25] Howe, C.L., Keller III, T.C.S., Mooseker, M.S. and Wasserman, R.H. (1982) Proc. Natl. Acad. Sci. USA 79, 1134–1138.
[26] Miyamoto, E., Kuo, J.F. and Greengard, P. (1969) J. Biol. Chem. 244, 67395–64002.
[27] Hausamen, T.U., Helger, R., Rich, W. and Gross, W. (1967) Clin. Chim. Acta 15, 241–245.
[28] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[29] Howe, C.L. and Mooseker, M.S. (1983) J. Cell Biol. 97, 974–985.
[30] De Boland, A.R. and Norman, A. (1990) Endocrinology 127, 39–45.