Calmodulin may mediate 1,25-dihydroxyvitamin D-stimulated intestinal calcium transport

Daniel D Bikle, Scott Munson and James Chafouleas*

Department of Medicine (111N), VA Medical Center and University of California, 4150 Clement Street, San Francisco, CA 94121 and **Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030, USA*

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1,25-Dihydroxyvitamin D (1,25$(OH)_2$D) stimulated an increase in calmodulin content in chick duodenal brush border membranes in parallel with an increase in duodenal calcium transport in vivo and calcium uptake by brush border membrane vesicles (BBMV) in vitro. The increase in calcium uptake by BBMV was blocked by specific calmodulin antagonists. These results suggest that calmodulin mediates 1,25$(OH)_2$D-stimulated calcium movement across the brush border membrane.

Calcium transport *Intestine* *Brush border membrane* *1,25-Dihydroxyvitamin D* *Calmodulin* *Calmodulin antagonist*

1. INTRODUCTION

The biologically active vitamin D metabolite 1,25-dihydroxyvitamin D [1,25$(OH)_2$D] administered in vivo increases calcium movement across the intestinal brush border membrane by a mechanism that appears to be independent of its ability to induce new proteins such as the vitamin D-dependent calcium-binding protein (CaBP) [1,2]. Another calcium-binding protein, calmodulin, is also found in the intestinal epithelium [3] and may play a role in intestinal calcium transport [4]. Total calmodulin concentrations in the intestine are not increased by 1,25$(OH)_2$D [3]. We observed that 1,25$(OH)_2$D stimulated an increase in calmodulin content in chick duodenal brush border membranes (without increasing total cellular levels) in parallel with an increase in duodenal calcium transport in vivo and calcium uptake by brush border membrane vesicles (BBMV) in vitro. The increase in calcium uptake by BBMV was blocked by specific calmodulin antagonists. These results suggest that 1,25$(OH)_2$D leads to a redistribution of calmodulin to the brush border membrane, permitting calmodulin to mediate 1,25$(OH)_2$D-stimulated calcium movement across the brush border membrane.

2. MATERIALS AND METHODS

White Leghorn cockerels were raised from hatching for 6 weeks on a vitamin D-deficient diet containing 1% Ca, 0.65% P. The 1,25$(OH)_2$D (gift from M.R. Uskokovic, Hoffmann-La Roche, Nutley, NJ) was given orally in doses of 625 pmol in 100 μl propylene glycol. Calcium transport in vivo was measured by the in situ duodenal loop technique [5]; 300 μl of a buffered solution containing 3 μCi ^{45}Ca and 3 mM calcium was injected into the proximal 5 cm of duodenum, which was ligated at both ends but in which the blood supply was preserved. After 15 min blood was obtained by cardiac puncture, and the ^{45}Ca level in serum was determined by liquid scintillation spectroscopy. BBMV were prepared as in [6] using the pooled duodenal mucosae of 4 chicks for each preparation. This method results in 10–15-fold purification of the brush border membrane markers sucrase and alkaline phosphatase in comparison to the initial homogenate. Calcium uptake

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by BBMV was measured as follows: 0.5 mg BBMV protein/ml was incubated in buffered solutions containing 10 μCi ^{45}Ca/ml and 2.5 mM calcium [7]. No ATP was present in this incubation medium. Extravesicular calcium was removed in the course of eluting the BBMV through a Dowex cation-exchange column, and the intravesicular calcium was quantitated by liquid scintillation spectroscopy. Calmodulin levels in BBMV were evaluated by radioimmunoassay [8] of BBMV extracts, which were prepared by heating the BBMV to 90°C for 5 min in 125 mM boric acid, 5 mM EGTA, 75 mM NaCl (pH 8.4).

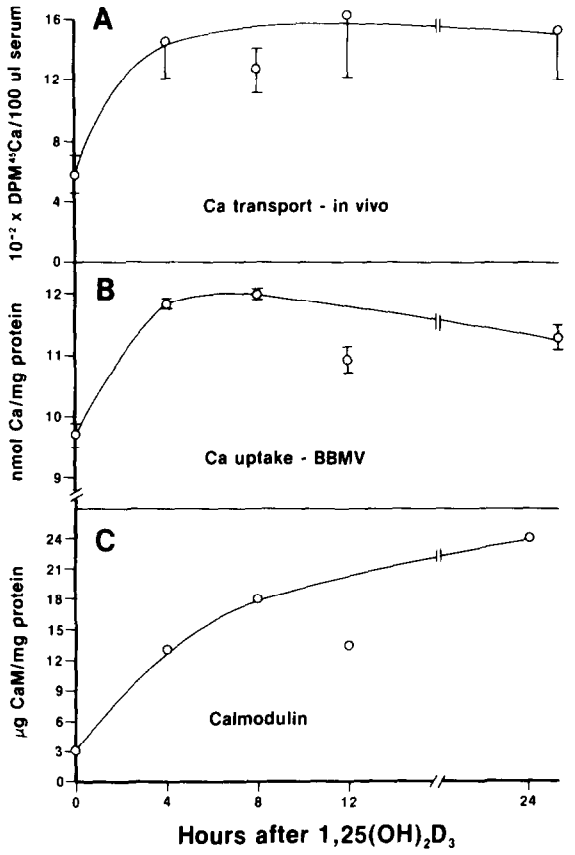

Fig.1. Calcium transport by chick duodenal loops in situ (A), calcium accumulation by chick duodenal brush border membrane vesicles (BBMV) (B), and calmodulin content of BBMV (C) at different times after the administration of 625 pmol 1,25(OH)$_2$D$_3$ to vitamin D-deficient chicks. The calcium transport in vivo measures the appearance of ^{45}Ca in the serum 15 min after injection into the proximal duodenum. Results are expressed as mean ± SE for each group of 4 chicks. Calcium uptake by BBMV was measured after incubation of BBMV in 2.5 mM calcium for 60 min at 25°C. Each BBMV preparation used the mucosae from 4 chicks. Results are expressed as mean ± range of duplicate determinations. Calmodulin (CaM) content was measured by radioimmunoassay. Each point is the mean of triplicate determinations, each of which was within 5% of the mean. * $P < 0.05$ *vs* values for controls.

3. RESULTS AND DISCUSSION

Administration of 1,25(OH)$_2$D to vitamin D-deficient chicks results in more than a 2-fold increase in duodenal calcium transport (from 590 ± 150 to 1440 ± 250 dpm, mean ± SE) by 4 h (fig.1A). The increase in calcium transport in vivo is paralleled by a 22% increase in the ability of BBMV to accumulate calcium (from 9.7 ± 0.2 to 11.8 ± 0.1 nmol Ca/mg protein, mean ± SE) (fig.1B) and a 4-fold increase in calmodulin levels

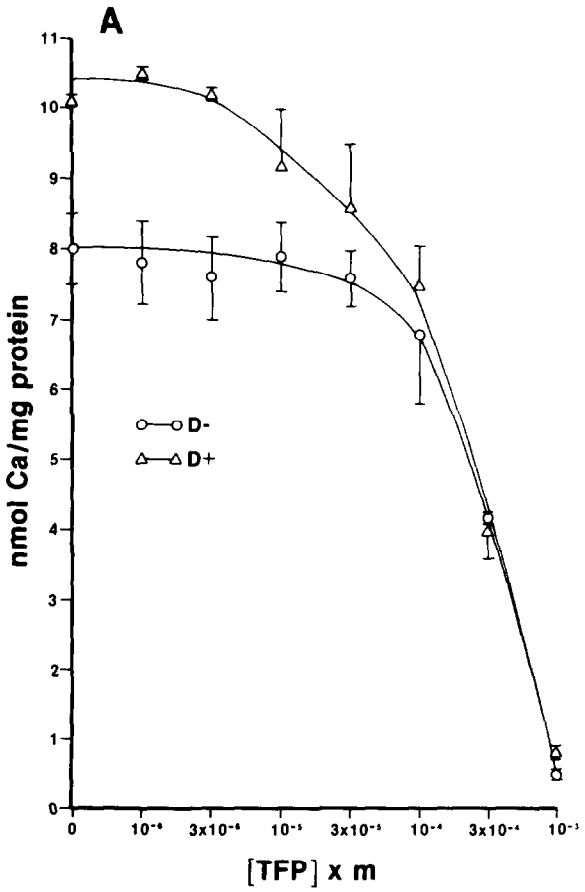

Fig. 2A. Legend on following page.

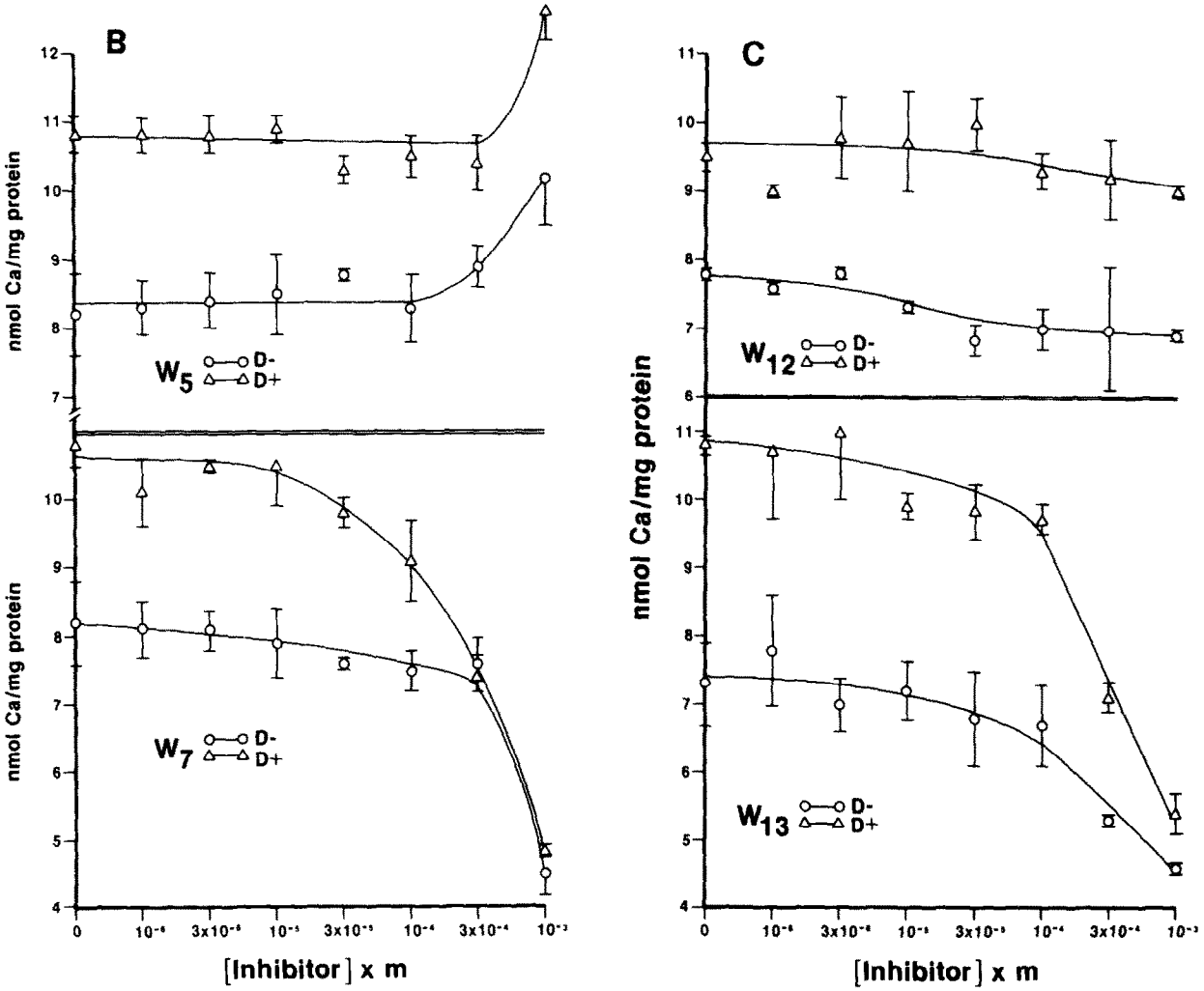

Fig.2. The effects of trifluoperazine (TFP) (A), W_5 and W_7 (B), and W_{12} and W_{13} (C) on calcium uptake by BBMV from vitamin D-deficient chicks (D−) and hatchmates that were given 625 pmol $1,25(OH)_2D$ 9 h before death (D+). TFP, W_{12} and W_{13} were prepared in water; W_5 and W_7 were prepared in ethanol. In the experiment with W_5 and W_7, final concentrations of ethanol in the incubation medium were 1% for $0–10^{-4}$ M inhibitor, 3% for 3×10^{-4} M inhibitor, and 10% for 10^{-3} M inhibitor. No ethanol was present in the TFP, W_{12} and W_{13} experiments. Control experiments with 0–10% ethanol, which were run simultaneously, indicated that calcium uptake was stimulated to the same extent by 3 and 10% ethanol as it was by 3×10^{-4} and 10^{-3} M W_5, respectively, indicating that ethanol, not W_5, accounted for the stimulation observed.

(from 3.3 to 13 μg CaM/mg protein) (fig.1C) over the same 4 h period. Calmodulin levels increased further after 4 h in this experiment, although no further increase in calcium transport in vivo or calcium uptake by BBMV in vitro was observed.

The ability of specific calmodulin antagonists [9] to block calcium uptake by BBMV was evaluated. BBMV prepared from vitamin D-deficient chicks (D-BBMV) were compared directly to BBMV prepared from vitamin D-deficient chicks given $1,25(OH)_2D$ 9 h before death (D+ BBMV). In these experiments (fig.2) the D+ BBMV accumulated 30% more calcium than did the D− BBMV (mean total difference, 2.4 nmol Ca/mg protein). The D+ BBMV had 17 μg/mg protein more calmodulin than did the D− BBMV,

although total cellular concentrations were similar: 9.2 ± 1.2 μg/mg protein in D− homogenates and 8.7 ± 1.2 μg/mg protein in D+ homogenates (mean ± SE). Trifluoperazine (TFP), *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W_7), its dechlorinated derivative W_5, *N*-(4-aminobutyl)-5-chloro-1-naphthalenesulfonamide (W_{13}), and its dechlorinated derivative W_{12} were added to the incubate 15 min before the addition of calcium. In other systems W_5 and W_{12} have little potency in antagonizing calmodulin action compared to W_7 and W_{13} [9]. However, W_5 and W_{12} have a lipophilicity similar to W_7 and W_{13} and serve as good controls for the specificity of W_7 and W_{13} in blocking calmodulin function.

TFP (fig.2A), W_7 (fig.2B) and W_{13} (fig.2C) inhibited calcium uptake, especially by D+ BBMV; the difference in calcium uptake between D+ and D− BBMV was obliterated at 10^{-4} M TFP and 3×10^{-4} M W_7, doses that only slightly inhibited calcium uptake by D− BBMV. The dechlorinated analogues of W_7 and W_{13} (W_5 and W_{12}, respectively) had little effect on calcium uptake by either D+ or D− BBMV (fig.2B,C). Although there was an apparent increase in calcium uptake at 3×10^{-4} and 10^{-3} M W_5 (fig.2B), this was due to the ethanol used as vehicle, not the drug (not shown); the increase in ethanol from 1 to 10% was required to maintain the higher concentrations of W_5 and W_7 in solution. TFP, W_{12} and W_{13} are water soluble and did not require the ethanol vehicle. At concentrations greater than 10^{-4} M, TFP, W_7 and W_{13} inhibited calcium uptake by both the D+ and D− BBMV. These results suggest that calmodulin in the D− BBMV is less sensitive to the calmodulin antagonists, perhaps because the calmodulin in the D− BBMV is less accessible to exogenously added calmodulin antagonists than is the calmodulin that appears in the BBMV following $1,25(OH)_2D$ administration.

The difference in calmodulin content between D− and D+ BBMV in this experiment was 17 μg/mg protein. This amount of calmodulin, if fully saturated with calcium, would bind 4 nmol Ca/mg BBMV protein (based on an M_r for calmodulin of 17000 and 4 calcium-binding sites per molecule [10]). This figure approximates the difference (2.4 nmol Ca/mg protein) in calcium uptake by D+ and D− BBMV. This difference in calmodulin content, then, could underlie the difference in calcium uptake between D+ and D− BBMV. However, the calmodulin antagonists TFP, W_7 and W_{13}, at 10^{-4} M, did not block calcium binding to calmodulin in vitro in the absence of BBMV (not shown). Therefore, calmodulin appears to enhance BBMV uptake of calcium by a mechanism other than its ability to bind calcium.

At present, the mechanism by which calmodulin enhances calcium uptake by BBMV remains uncertain. Calcium uptake by BBMV does not require ATP and, therefore, presumably does not involve a Ca^{2+}-ATPase. Perhaps calmodulin interacts with a calcium carrier or channel in the membrane to facilitate calcium movement through the membrane down its electrochemical gradient. It may be necessary to determine the function of the calmodulin-binding sites in the BBMV in order to understand the mechanism by which calmodulin increases calcium movement across the brush border membrane.

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