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Hormone Mediated Transport of Calcium and Phosphate in Polarized Epithelial Cells

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HORMONE MEDIATED TRANSPORT OF CALCIUM AND PHOSPHATE IN POLARIZED EPITHELIAL CELLS

by

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ABSTRACT

Hormone Mediated Transport of Calcium and Phosphate in

Polarized Epithelial Cells

by

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The effects of $1,25(OH)_2D_3$, PTH and $25(OH)D_3$ on phosphate or calcium uptake were studied in cultured, adherent chick enterocytes over a period of 10 min after hormone addition. Time course studies of cells treated with 130 pM $1,25(OH)_2D_3$ showed an increase in ^{32}P uptake as early as 3 min. Similar studies with 65 pM bPTH(1-34) resulted in an increase in ^{45}Ca uptake only if the cells had been cultured in serum. $25(OH)D_3$, which is not firmly established as an active metabolite of vitamin D, was shown to increase ^{45}Ca uptake within 5 min at a 100 nM concentration.

Analyses of signal transduction events involving each hormone were undertaken using PKC and PKA inhibitors, chelerythrine and Rp-cAMP, respectively. In the presence of PKC inhibitor and $1,25(OH)_2D_3$ elevated ^{32}P levels were apparent; however, further investigations involving efflux studies showed PKC inhibition of ^{32}P extrusion in the presence or absence of hormone. On the other hand, suppression of the PKA pathway stimulated an increase in $1,25(OH)_2D_3$–mediated ^{32}P uptake. Preincubation of

enterocytes with Ab099 against a putative membrane receptor for $1,25(OH)_2D_3$ abolished steroid-stimulated ^{32}P uptake.

While PKC inhibition had no effect on ^{45}Ca uptake in enterocytes exposed to 65 pM bPTH(1-34) in serum, pretreatment with PKA inhibitor resulted in ^{45}Ca levels relatively close to basal levels. Cells pretreated with PKC inhibitor and exposed to $25(OH)D_3$ demonstrated no changes in ^{45}Ca levels, whereas inhibition of PKA induced decreased ^{45}Ca levels after 10 min of incubation.

In equivalent time course studies of membrane trafficking using confocal microscopy, potential vectorial transport initiated by each hormone was analyzed with agonist alone or in the presence of PKC and PKA inhibitors. In addition $1,25(OH)_2D_3$ was tested in the presence of Ab099 against its putative membrane receptor. Visualization of these experiments using the endocytotic marker dye, FM 1-43, demonstrated that hormone-mediated membrane trafficking is rapid enough to contribute to ion transport. These results also suggest that vectorial vesicular transport mechanisms were involved to some extent in response to each hormone. Moreover, the pattern for membrane trafficking was different for each agonist.

These combined results indicate that adherent chick enterocytes demonstrate hormone-mediated uptake that occurs more rapidly than cells in suspension or in perfusion studies. This research supports previous studies that identify $25(OH)D_3$ as an active vitamin D metabolite. The PKA signal transduction pathway is a possible mechanism for PTH- and $25(OH)D_3$-mediated increases in ^{45}Ca. In addition, a central role for the basal lateral membrane receptor protein, $1,25(OH)_2D_3$MARRS-bp, in $1,25(OH)_2D_3$–mediated ^{32}P uptake is supported. Confocal imaging suggests that the

transport mechanism for phosphate or calcium ions in the presence of these hormones involves vesicular carriers.

(73 pages)

To Mattie and Carolyn

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First and foremost, I would like to acknowledge God's unyielding presence in my life. With His Guidance, I have been exposed to limitless opportunities and have met many individuals who have facilitated the intellectual and personal growth required to succeed at this level.

I would like to dedicate this manuscript to my grandmother, Mattie Bell Mc Bride, whose passing inspired me to pursue science, and my mother, Carolyn A. Sterling, who continually motivated me to follow my dreams and to always strive to improve.

I want to thank my family and friends for their support and encouragement, especially my husband, Robert G. Peters, Jr. I would also like to thank all the individuals who challenged me to follow "the road less traveled," with special thanks to Mrs. Payne, Sr. Ignatia O'Rourke, and Piper D. Griffin.

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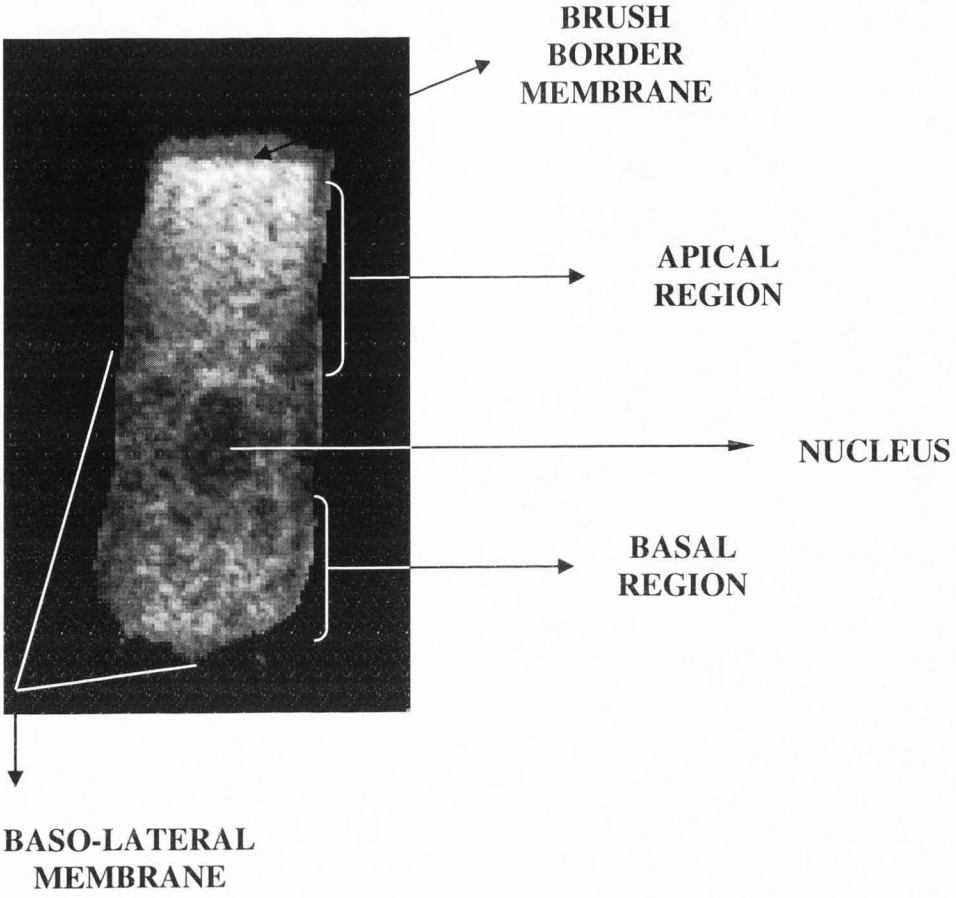


LIST OF ABBREVIATIONS AND DEFINITIONS

Ab099.....antibody against the $1,25D_3$-MARRS protein

Apical region.....region in intestinal epithelial cells that extends from the base of the microvilli of the brush border to just above the nucleus (see micrograph, p. viii).

Basal region.....region in intestinal epithelial cells that extends from the base of the nucleus to the baso-lateral membrane (see micrograph, p. viii).

bPTH(1-34).....bovine parathyroid hormone fragment comprised of N-terminal amino acids 1-34

Baso-lateral membrane .....membrane of intestinal epithelial cells that excludes the microvillus brush border membrane (see micrograph, p. viii).

$1,25(OH)_2D_3$.....1,25-dihydroxyvitamin D_3

$1,25D_3$-MARRS protein .....1,25-dihydroxyvitamin D_3-**m**embrane **a**ssociated **r**apid **r**esponse **s**teroid binding protein

PKA.....protein kinase A

PKC.....protein kinase C

$25(OH)D_3$.....25-hydroxyvitamin D_3

$24,25(OH)_2D_3$.....24,25-dihydroxyvitamin D_3

CHAPTER I

INTRODUCTION

In polarized epithelial cells calcium and phosphate transport are proposed to occur in a three-step process: movement from the intestinal lumen across the brush border of intestinal epithelial cells, transcellular transport from the brush border to the baso-lateral membrane, and finally extrusion. In the instance of both calcium and phosphate transport, transporters are proposed to exist at the brush border and baso-lateral membrane that are specific to each ion. On the other hand, the method of transcellular transport is still debated. Currently, the two proposed models that address the method of transcellular movement are facilitated diffusion and vesicular transport.

In the facilitated diffusion model, transport proteins bind to the ions and accelerate their transport across the cells to the baso-lateral region. This model proposes that ions bound to transport proteins exhibit characteristics similar to enzyme kinetics, where binding is saturable and transport occurs 10^3 times faster than simple diffusion. Slepchenko and Bronner[1] have illustrated these aspects of facilitated diffusion through mathematical modeling that identifies the transport protein, calbindin, as the rate-limiting step in calcium transport. On the contrary, Fleet et al.[2] report that elevated calbindin levels in Caco-2 cells are not directly correlated with higher net Ca^{2+} calcium transport, suggesting that other mechanisms may be involved.

An alternative to the facilitated diffusion model is the vesicular transport model in which the ion is endocytosed at the base of the brush border and exocytosed near the baso-lateral membrane; in this model, the presence of calbindin may act as a buffering molecule when associated with vesicles containing calcium. Findings by Nemere and

Norman[3] in perfused duodenal loops suggest that calcium transport may occur by the vesicular transport model. Several studies[4,5,6] report lysosomal fractions containing high calcium and calbindin levels, but further investigations will be necessary to determine the different roles that calbindin may exhibit in calcium transport. In comparison, Wasserman[7] proposed that transcellular phosphate movement occurs by a vesicular route that is independent of calcium transport. Karsenty et al.[8] support Wasserman's findings and suggest that phosphate uptake parallels structural lipid modifications of the plasma membrane, indicating an endocytotic mechanism. Although these studies provide evidence to support vesicular transport, there is still concern over the ability of calcium and/or phosphate transport to occur rapidly enough to account for the speed of ion uptake that are observed in the presence of hormone. Although studies in isolated human fibroblast lysosomes have demonstrated that calcium uptake reaches a steady state by 10 min[6] and phosphate uptake reaches its steady state by 8-10 min,[9] more studies must be undertaken to determine the mechanisms that are involved.

CHAPTER II

REVIEW OF LITERATURE

The body requires calcium for various functions including muscle contraction, blood clotting, nerve impulse propagation and skeletal formation; phosphate is required for various functions that include but are not limited to energy transfer, nucleic acid composition, acid-base balance, and skeletal formation. As the two most abundant elements in the body, 99% of the calcium and 85% of the phosphate are used primarily for bone mineralization. During the process of bone mineralization, calcium and phosphate are incorporated into the organic matrix to form calcified bone tissue known as hydroxyapatite. The evolution of these minerals into bone tissue involves a series of steps that have been identified as either intramembranous ossification for flat bone formation or endochondral ossification for long bone formation. In both processes, mesenchymal cells from embryonic connective tissue proliferate and differentiate into preosteoblasts or prechondrocytes. In intramembraneous ossification, the differentiated cells form preosteoblasts that eventually mature into osteoblasts. On the other hand, the differentiated cells in endochondral ossification form prechondrocblasts, which mature into chondrocytes. Magne et al.[10] demonstrated that these chondroblasts become hypertrophic and calcify in the presence of increased phosphate and calcium concentrations. Consequently, the hydroxyapatite that composes bone tissue is formed. The phosphate and calcium stored in this bone matrix undergoes continual modeling and remodeling, depending on both absorption of the ions in the intestine and their circulating serum levels. In order to maintain bone density, it is very important for adequate amounts of phosphate and calcium to be absorbed by the intestine; any deficiency that

compromises absorption will cause the bone matrix to erode to compensate for circulating levels of these ions that are necessary for other cell functions.

It has been demonstrated in previous research that calcium and phosphate are absorbed from the diet through a pathway across intestinal cells and may be eventually delivered to the bloodstream. There have been several theories that attempt to explain the movement of these minerals across the epithelium of the duodenum to their final destination. The transport of calcium and phosphate is regulated by the presence and/or concentration of hormones, such as 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$], 25-hydroxyvitamin D_3 [$25(OH)D_3$] and parathyroid hormone (PTH), which maintain homeostatic concentrations of these ions.

Proposed Models for Transport Mechanisms

Calcium Transport

Two general theories are currently being used to explain calcium transport across polarized epithelial cells. The first theory formulated to explain the $1,25(OH)_2D_3$-stimulated movement of calcium across epithelial cells is the facilitated diffusion model.[1] In this model, calcium uptake was postulated to occur at the apical membrane by a transporter or channel. Calcium is then bound by a calcium-binding protein called calbindin to facilitate its diffusion across the cytoplasm of the cell. Wasserman and Taylor[11,12] discovered the presence of calbindin and proposed its role in translocating calcium within intestinal cells of chicks. Calcium is then extruded extruded by a Ca^{2+}-ATPase located at the baso-lateral membrane.[13] Although this theory is widely

accepted, there is evidence that facilitated diffusion may not occur quickly enough to account for the rapid transcellular transport of calcium.[14]

The alternative theory postulates that vesicles containing calbindin [15] sequester calcium at the apical membrane, after recognition of the cation by a transporter, and use cytoskeletal elements in the transcellular movement of the ion in the intestine. Vesicular transport has also been postulated in the chorioallantoic membrane from egg shells. [16] Using electron microscopy, Jande and Brewer[17] proposed an active role of lysosomes in the transcellular transport of calcium in rat intestinal cells. Similar transcellular localizations of calcium in either rat and /or chick intestinal cells were also verified by Warner and Coleman[4] and Davis et al.[18] using microscopic techniques. Bikle et al.[19] found calcium to be sequestered in vesicles following transport conditions in chick enterocytes using autoradiography. Confirmation using biochemical techniques was reported by Nemere et al.[20] Moreover, using a combination of differential and Percoll gradient centrifugation, vesicular calcium levels were found to closely parallel $1,25(OH)_2D_3$-stimulated calcium absorption in rachitic chicks with respect to time course[21] and dose response effects.[22] The vesicular route has been shown to incorporate endocytotic and transcytotic organelles and/or lysosomes as candidates for cellular calcium carriers.[19,22] Support for lysosomes as calcium ion carriers is further cited by Rodriguez et al.[23] in their findings with fibroblast and epithelial cells, which suggest that vesicular pathway is involved.

Phosphate Transport

Phosphate uptake has been shown to occur through a Na/PO_4 transporter at the apical membrane.[24,25] Internalization of phosphate occurs in the lysosomal

compartment[5,26] by a lysosomal ATPase.[27] Lysosomal sequestration of both calcium and phosphate has been reported for fibroblasts[6,27] as well as for enterocytes.[5,20,26] Once transported to the baso-lateral membrane, it has been postulated by [24] that an anion/ phosphate exchange occurs.

Hormonal Effects on Calcium and Phosphate Transport

Vitamin D is a fat-soluble vitamin that is also known as cholecalciferol (in animals) and ergocalciferol (in yeast and plants) in its inactive state. This prohormone is either directly synthesized by the skin through a photochemical process or can be obtained from vitamin D-enriched foods. Vitamin D is activated by the liver, where it is hydroxylated at carbon-25, and the kidney, where $25(OH)D_3$ is either hydroxylated at carbon-1 or carbon-24.

Thus far, the three known active metabolites are $25(OH)D_3$; [28,29] $1,25(OH)_2D_3$;[30] and $24,25(OH)_2D_3$.[26,31-33] While $25(OH)D_3$ causes an increase in calcium uptake in rat duodena,[34] it has also been shown to increase calcium transport in the perfused duodenal loops of chicks.[29] In isolated chick enterocytes and perfused duodenal loops, Zhao and Nemere[35] have reported that $1,25(OH)_2D_3$ increases phosphate uptake and transport. On the other hand, $24,25(OH)_2D_3$ has an inhibitory effect when administered in the presence of $1,25(OH)_2D_3$.[26,36]

Parathyroid hormone is a polypeptide chain that is released from the parathyroid glands in direct response to low circulating levels of calcium.[37] This release of parathyroid hormone stimulates changes in serum calcium through three target tissues, the intestine, kidneys and bone. Of these, skeletal tissue is the primary source for

increasing serum calcium levels through bone resorption. Circulating PTH levels interact with $1,25(OH)_2D_3$ in a feedback mechanism that causes the demineralization of bone to increase serum calcium and phosphate levels. As with $25(OH)D_3$, PTH has also been found to increase calcium transport in perfused duodenal loops, [38] and calcium uptake in intestinal epithelial cells from rats.[39]

Proposed Signal Transduction Pathways

In previous literature, PKC and PKA signal transduction pathways have been implicated as possible mechanisms for calcium and phosphate transport in intestinal cells. deBoland and Norman[40] have shown that both the PKA and PKC stimulators mimic $1,25(OH)_2D_3$ activation of calcium transport in perfused duodenal loops. Larsson and Nemere[41] reported that the biphasic dose-response curve for $1,25(OH)_2D_3$ stimulated increases in PKC activity was comparable to observed increases in phosphate transport in that each was optimal at 130 pM-300 pM steroid, with inhibition at 650 pM hormone. By comparison the dose-response curve for hormone stimulated increases in PKA activity and calcium transport exhibited a maximum at 650 pM $1,25(OH)_2D_3$. Zhao and Nemere[35] demonstrated that phorbol ester, an activator of PKC, stimulates phosphate uptake and transport in the presence of $1,25(OH)_2D_3$ in both perfused and isolated intestinal cells; forskolin, an activator of PKA, on the other hand, does not stimulate phosphate uptake or transport. In the presence of $25(OH)D_3$, a very brief stimulation of PKA activity was observed by Phadnis and Nemere. [29] In addition, forskolin treatment of intestinal epithelial cell suspensions stimulated ^{45}Ca uptake, whereas 100nM $25(OH)D_3$ apparently stimulated ^{45}Ca extrusion. These combined findings suggest that the PKA signaling is involved in calcium uptake that is observed in presence of

$1,25(OH)_2D_3$, PTH and possibly $25(OH)D_3$. This evidence also suggests that PKC is a possible mechanistic pathway for phosphate uptake that is observed in the presence of $1,25(OH)_2D_3$. Each of these pathways have been implicated in some aspect of vesicular trafficking; Choi et al.[42] have demonstrated in rat mast cells that PKA is involved in the translocation of vesicles and PKC regulates fusion of these vesicles with the plasma membrane.

This research was designed to test the hypothesis that mechanisms for hormone mediated calcium and phosphate transport across polarized intestinal epithelial cells are facilitated by vesicular trafficking. The specific objectives were to:

1.) Test adherent chick intestinal cells for their response to the hormones $25(OH)D_3$, $1,25(OH)_2D_3$, or PTH in the presence of ^{45}Ca or ^{32}P and determine how they compare to observations for cells in suspension;

2.) Understand which signal transduction pathways are involved with phosphate or calcium uptake in polarized epithelial cells by using inhibitors of PKA or PKC in conjunction with agonist.

3.) Evaluate the contribution of vesicular compartments to the vectorial transport of calcium and phosphate using confocal microscopy in the presence of agonist with or without inhibitors; and

4.) Identify the involvement of the $1,25(OH)_2D_3$ **m**embrane-**a**ssociated **r**apid **r**esponse **s**teroid **b**inding **p**rotein (MARRS-bp) in phosphate uptake mediated by the steroid hormone.

CHAPTER III

MATERIALS AND METHODS

Animals and Surgical Procedures

All surgical procedures were approved by the Institutional Animal Use and Care Committee at Utah State University (Logan, UT). White leghorn cockerels (Privett Hatchery; Portales, NM) were obtained on the day of hatch and raised for 3-7 weeks on a commercially available vitamin D-replete diet (Nutrena Feeds; Murray, UT). On the day of use, chicks were anesthetized with chloropent (0.3 ml/100g body weight), the duodenal loop was surgically removed to ice-cold 0.9% saline solution and chilled for 15 min. The pancreas was excised from the duodenal loop and discarded. The duodenal loop was everted and rinsed with chilled saline solution.

Cell Isolation

The chick enterocytes were isolated with citrate chelation media[35,43] adjusted to pH 5.0 to promote viability and retention of morphology. [44] The cells were collected by low speed centrifugation (500 **g**, 5 min, 4°C), resuspended in a small volume of Gey's balanced salt solution (GBSS, containing 119 mM NaCl, 4.96 mM KCl, 0.22 mM KH_2PO_4, 0.84 mM $NaHPO_4$, 1.03 mM $MgCl_2 \bullet 6H_2O$, 0.28 mM $MgSO_4 \bullet 7H_2O$, 0.9 mM $CaCl_2$, pH 7.3), and transported on ice to the Center for Integrated Biosystems (Logan, UT). Aliquots of the cell suspension (1 ml) were pipetted into 35 mm plastic petri dishes (Falcon, Scientific Products; Franklin Lakes, NJ) containing 3 ml of RPMI-1640 medium and antibiotics [100 units/ml penicillin, 100 mg/ml streptomycin (both from Sigma Chemical Co; St. Louis, MO)]. The cells were incubated for 24 hours without serum to

promote cell adhesion. In some experiments, media were changed to contain 10% fetal bovine serum (Hyclone; Logan, UT) with antibiotics and cultured for an additional 24 hours at 37°C with 5% CO_2/ 95% air.

Time Course of Radionuclide Uptake

After incubation as described above, the medium in each dish were aspirated and replaced with GBSS containing radionuclide for uptake studies. After each dish was exposed to GBSS with radionuclide (1 μCi/ml $^{45}CaCl_2$ or 2 μCi/ml $H_3{}^{32}PO_4$) for 10 min, dishes for the zero time point were washed three times with 4 ml of ice cold GBSS, and treated with lysis buffer (10 mM Tris, 1.25 mM EDTA, 2 mM dithiothreitol, pH 7.4 {TED} containing 0.1% Triton X-100). The remaining dishes were treated with 0.01% ethanol for vehicle controls or appropriate test substances. The final concentrations for each test substance were 130 pM $1,25(OH)_2D_3$, 100 nM $25(OH)_2D_3$, or 65 pM bovinePTH 1-34 fragment [bPTH(1-34); Sigma]. At specific time intervals (1-, 3-, 5-, 7-, and 10-min after additions) the media were aspirated and the cells washed and then lysed in preparation for protein determination by the Bradford Assay (BioRad; Hercules, CA) and radionuclide measurement by liquid scintillation spectrophotometry.

An aliquot of cell lysate was combined with 3 ml of liquid scintillation cocktail. Radionuclide uptake was then measured using a liquid scintillation spectrophotometer and expressed as cpm. The cpm in each sample was related to protein levels in the corresponding sample to yield specific activity. For time course studies, basal uptake levels were used to normalize sample values obtained during the treated phase.

Time Course of Radionuclide Efflux

Cells from a single chick were resuspended in 40 ml of medium and 3 ml seeded into each petri dish. After incubation overnight as described above, half of the dishes were treated with 0.66 μM chelerythrine or 3 μM Rp cAMP, and incubated for 3 h or 0.5 h, respectively, prior to experimentation. Media and non-adherent cells were removed, and 2 ml of GBSS containing either 2 μCi/ml of $H_3{}^{32}PO_4$ or 1 μCi/ml of $^{45}CaCl_2$ added for a 7 min uptake period. Pilot studies indicated that uptake for either radionuclide achieves a plateau between 5-10 min. Thereafter, radioactive media were removed, the cells rinsed with two 3-ml aliquots of GBSS, and a fresh 3-ml aliquot of GBSS added at T=0 min. Immediately thereafter, a 100 μl sample was removed and vehicle or hormone added. Sampling of the media continued at 1-, 3-, 5-, 7-, and 10-min intervals. Samples were pipetted directly into scintillation vials containing fluor for counting. All cpm were related to corresponding values at 0 min for normalization.

Inhibitor Studies

To assess the role of PKC, adherent cells were preincubated with or without 0.66 μM chelerythrine (Sigma) for 3-4 h[45] and then incubated with radionuclide with and without hormone as described above. To evaluate the contribution of PKA, cells were preincubated with or without 3 μM Rp-cAMP (Sigma) for 30-60 min[46] prior to introduction of isotope, followed by vehicle or hormone.

The role of the $1,25D_3$-MARRS protein was analyzed using a highly specific polyclonal antibody (Ab099) generated by the multiple antigenic peptide format to the N-terminal sequence of the protein (Center for Integrated BioSystems; Logan, Utah). For ^{32}P uptake studies, cells will be incubated as previously stated, as well as in the presence

of Ab099 (1/500) dilution for 5 min prior to addition of vehicle or 130 pM $1,25(OH)_2D_3$. Confocal microscopy was likewise performed on cells preincubated with Ab099 and then exposed to hormone.

Protein Determination

The protein content of epithelial cells at each time point was determined with the Bradford reagent (BioRad; Hercules, CA) using bovine γ-globulin as the standard.

Confocal Microscopy

Vesicular movement was examined using confocal microscopy using probes, which were internalized into the plasma membrane. A BioRad MRC 1024 laser-scanning confocal microscope system mounted in the Keller position and attached to a Nikon TE-200 microscope was used for confocal imaging. The krypton-argon laser produced 3 excitation lines of 488 nm, 568 nm, and 647 nm. The emission filter consisted of a 522/32 bandpass filter that collected all light between 506 –538 nm. Images were collected with BioRad LASERSHARP acquisition software, using a 60x oil immersion objective and analyzed for pixel intensity using Adobe Photoshop 6.0 (Adobe Systems, Inc; San Jose, CA).

Cells resuspended in RPMI were pipetted into German borosilicate, chambered glass coverslips (Lab-Tek; Fisher Scientific) containing 3-5 ml of RPMI-1640 media with anitbiotics at 37°C with 5% CO_2/ 95% air. FM 1-43 (Molecular Probes; Eugene, OR) was used to label endocytotic, or exocytotic vesicles; this label was excited by the 488 nm laser line with a maximum emission spectra at 625 nm. Lysotracker-red (Molecular Probes; Eugene, OR) was used for lysosomal visualization; this probe was excited at 568

nm laser line with a maximum emission spectra at 590 nm. The dyes were diluted into GBSS-0.1% BSA and the solution was then added to the cells. After a 15- to 30-min incubation with the dye, the medium was overlayed with GBSS-0.1% BSA only for vehicle or GBSS-0.1% BSA with test substances for treated cells. For inhibitor studies, cells were preincubated with 3 μM Rp-cAMP or 0.66 μM chelerythrine prior to addition of fluorescent label.

The images were then captured at 5-sec intervals for 10 min with BioRad LASERSHARP acquisition software, using a 60x oil immersion objective and analyzed for pixel intensity using Adobe Photoshop 6.0 (Adobe Systems, Inc; San Jose, CA).

Statistical Analysis

Values are expressed as mean ± SEM for the number of independent experiments indicated in the figure legends. The data were analyzed for significance using Student's t-test; significant differences were determined with a 95-97% probability.

CHAPTER IV

RESULTS

Effect of $1,25(OH)_2D_3$ on Phosphate Uptake

In isolated chick enterocytes, 130 pM $1,25(OH)_2D_3$ has been shown to stimulate phosphate uptake after 5 minutes.[35] In the current study, time course experiments on ^{32}P uptake in isolated cells were compared between $1,25(OH)_2D_3$ and controls. Figure 1 shows that 130 pM $1,25(OH)_2D_3$ stimulated an apparent increase in ^{32}P uptake within 1 min when compared to controls. In the controls, the corresponding ^{32}P treated/avg basal values (mean ± SEM) for T=1, 3, 5, 7 and 10 min were 0.71 ± 0.14, 0.45 ± 0.09, 0.50 ± 0.12, 0.47 ± 0.11, and 0.49 ± 0.11, respectively. After addition of $1,25(OH)_2D_3$, ^{32}P uptake increased by approximately 203%, 216%, 204% and 214% of controls at T=1, 3, 5, 7 and 10 min, respectively (n=5, $P<0.05$ for T=3-10 min).

Effect of $1,25(OH)_2D_3$ on Endocytotic Distribution of FM 1-43

An equivalent time course was used in studies using confocal microscopy with FM 1-43. This dye, which is nonfluorescent in an aqueous environment, fluoresces when associated with a membraneous environment, and thus can be used to monitor endocytosis. Images of control cells revealed bright fluorescence throughout the length of the cells, including the brush border (Fig. 2A). Solid areas of fluorescence appeared to be associated with the plasma membrane, and largely obscured punctate fluorescence (Fig. 2A). In pilot studies, fluorescent labeling remained unchanged for 10 min under control conditions. In subsequent studies, control conditions were applied for 50 sec of image capture.

Within 5 sec of $1,25(OH)_2D_3$ addition, a dramatic decrease in apical fluorescence was observed (Fig. 2B) with a further decrease at 10 sec (Fig. 2C). The intensity of punctate fluorescence increased markedly after 15 sec (Fig. 2D). These very rapid effects were observed in three independent experiments. In subsequent frames (Figs. 2F-2M), apical fluorescence appeared punctate while basal staining appeared continuous. Between 1 min and 1min 25 sec after $1,25(OH)_2D_3$ addition, the staining pattern became more punctate at supranuclear localizations (Figs. 2M-2R). A loss of overall

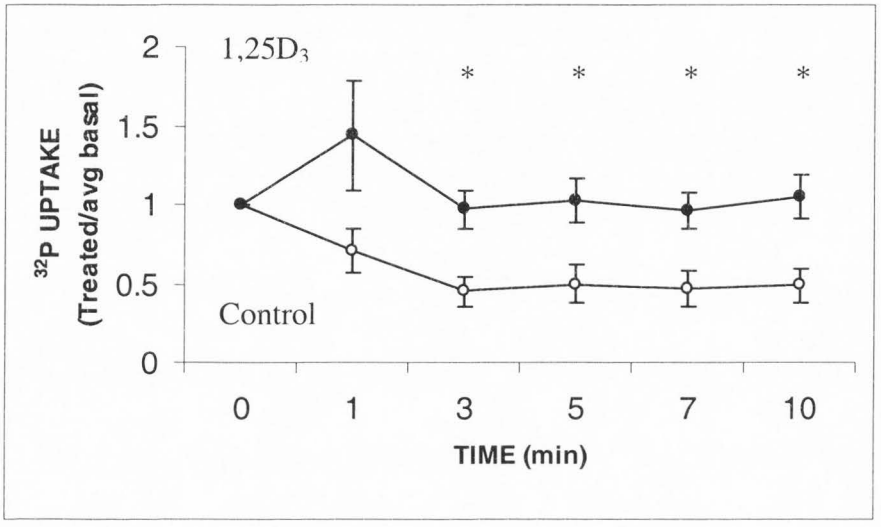


Fig. 1. Time course of $1,25(OH)_2D_3$-mediated ^{32}P uptake in isolated intestinal epithelial cells. Enterocytes were isolated by a chelation protocol, collected by low speed centrifugation, and resuspended in Gey's Balanced Salt Solution (GBSS). One-ml aliquots of cell suspensions were seeded into 35 mm Petri dishes containing 3 ml RPMI and incubated overnight (37°C with 5% CO2/95% air) to allow cells to adhere. Cells were treated with 130 pM $1,25(OH)_2D_3$ (●–●) or vehicle (0.01% ethanol, final concentration, ○–○), and washed with GBSS at the indicated times. After lyses in buffer containing 0.1% Triton X-100, aliquots were taken for determination of protein using the Bradford assay, cpm analysis by liquid scintillation spectrophotometry. Data were calculated as cpm/μg protein and then related to corresponding basal levels. Values represent mean ± SEM (n =6). Significant differences ($P<0.05$) were determined using the student t-test, are relative to corresponding controls, and are indicated by an asterisk (*).

fluorescence was again observed between 1 min 30 sec and 2 min 5 sec after hormone addition, followed by a dramatic increase in fluorescence 5 sec later (Fig. 2AA). A similar loss and return of fluorescence was observed around 3 min after hormone addition (data not shown). In pilot studies, at low resolution microscopy and in real time, this increase and decrease in fluorescent intensity occurred so rapidly that cells treated with $1,25(OH)_2D_3$, but not $25(OH)D_3$, appeared to flicker. For the remainder of the timecourse (3 min – 9 min after $1,25(OH)_2D_3$ addition), an overall increase in fluorescence was observed (Figs. 2BB, 2CC, 2DD, 2EE).

A second dye, Lyso-Tracker Red, was also tested in time course studies using confocal microscopy. This dye, which is nonfluorescent at a neutral pH, fluoresces when incorporated into acidic organelles. Studies with Lyso-Tracker Red suggested increased movement of labeled organelles after hormone (data not shown), but definitive patterns of movement were not evident. Similar inconclusive results were observed in enterocytes labeled with the dye and treated with PTH or $25(OH)D_3$ (data not shown).

Effects of bPTH(1-34) on Calcium Uptake

Previous studies have demonstrated that bPTH(1-34) increases calcium uptake in perfusion experiments within 12 min.[38] Pilot studies with enterocytes cultured in the absence of serum for 24 hr indicated a lack of responsiveness to PTH. However, when enterocytes were cultured for an additional 24 h in the prescence of 10% FBS, responsiveness returned. Figure 3 illustrates the effect of 65 pM bPTH (1-34) on calcium uptake in isolated enterocytes cultured with serum. Enhanced ^{45}Ca levels were noted as early as 1 min when compared to controls. ^{45}Ca uptake in bPTH(1-34)- treated cells was

significant at T=3, 5 and 10 min (P<0.02 to 0.05 relative to corresponding controls) with treated/avg basal levels of 1.33 ± 0.15, 1.39 ± 0.23, and 1.24 ± 0.11, respectively.

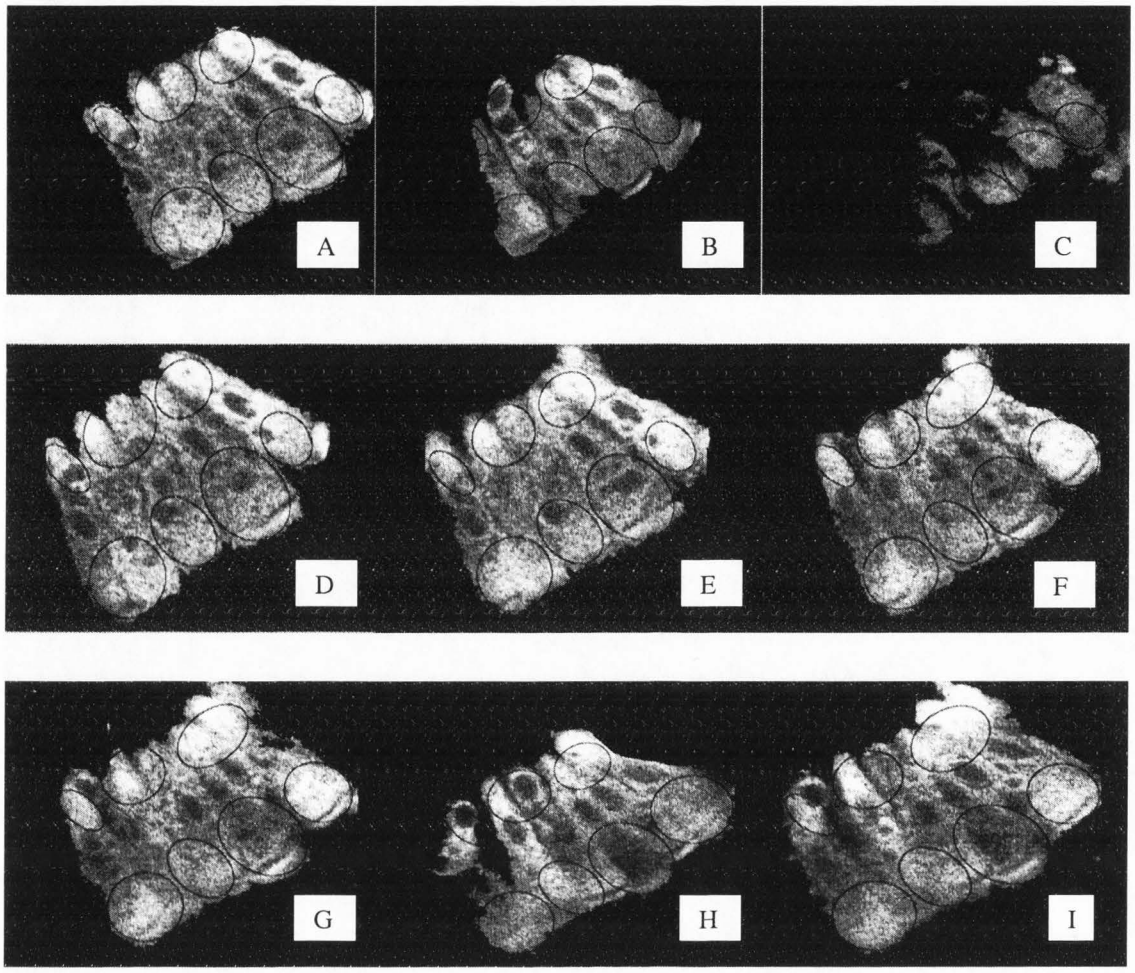
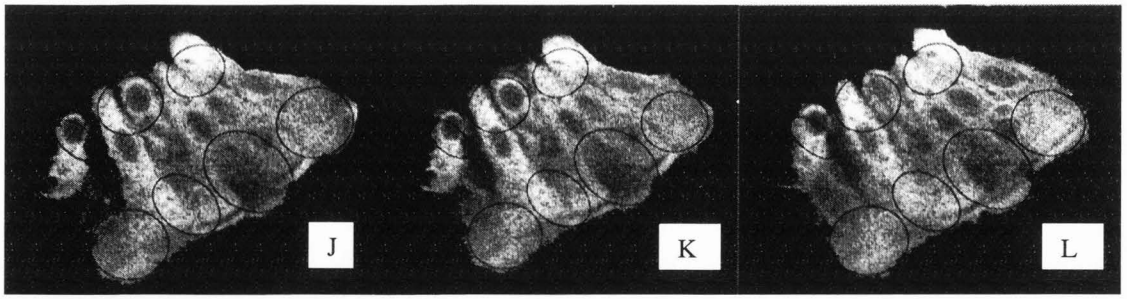


Fig. 2. Effect of $1,25(OH)_2D_3$ on endocytotic distribution of FM 1-43. Confocal images of cells labeled with FM1-43, then treated with $1,25(OH)_2D_3$. Micrographs are representative of three independent experiments. Cells were plated overnight in chambered coverslips. The dye was diluted into GBSS, which was then overlayed onto the cells. After incubation with the dye, GBSS containing hormone was added to the medium and images were captured at 5-sec intervals. The images were then captured at 5-sec intervals for 10 min with BioRad LASERSHARP acquisition software, using a 60x oil immersion objective and analyzed for pixel intensity using Adobe Photoshop 6.0. The emission and excitation wavelengths were 488 nm and 522, respectively. Areas indicated by circles, elipses, squares, or rectangles in this and subsequent images were analyzed for intensity. These values are summarized in tables presented in the appendix. (A) control; (B-AA) every 5 sec after hormone; (BB) 3 min; (CC) 5 min; (DD) 7 min; (EE) 9 min.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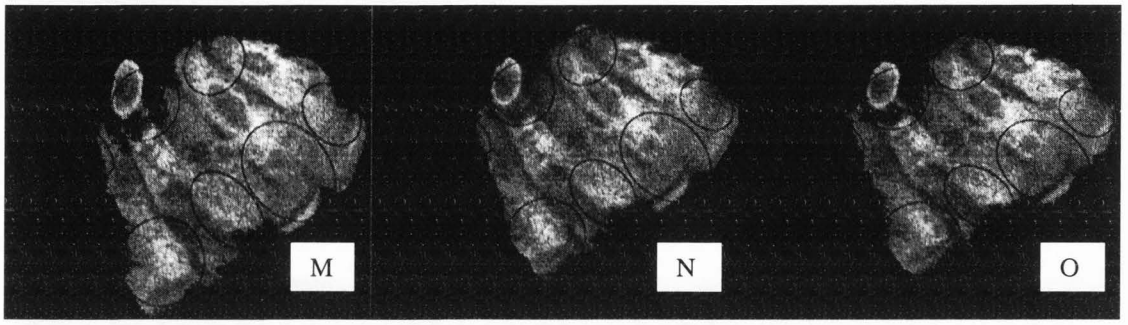
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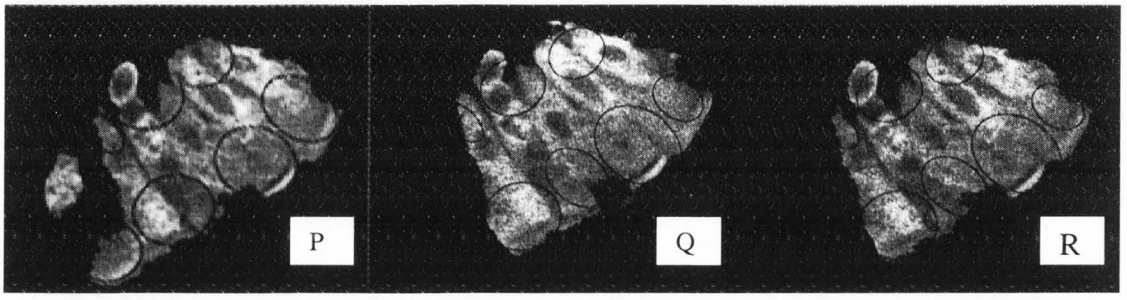
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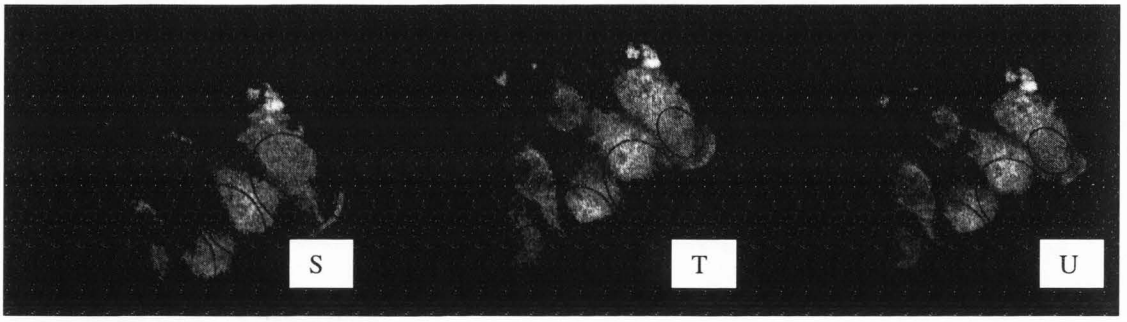
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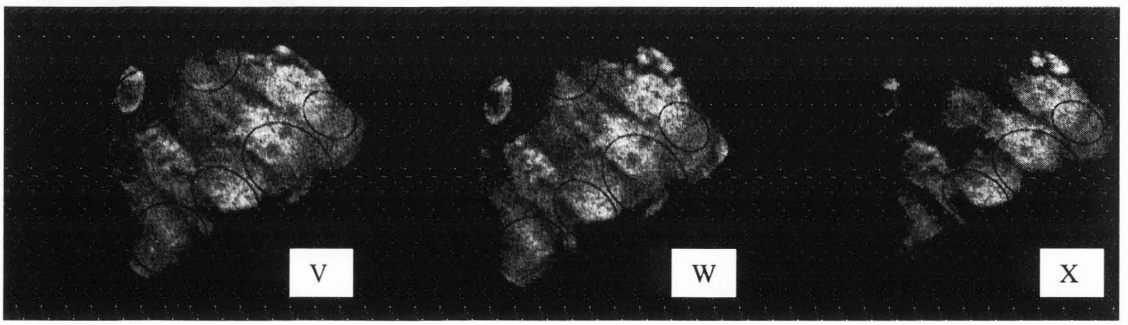
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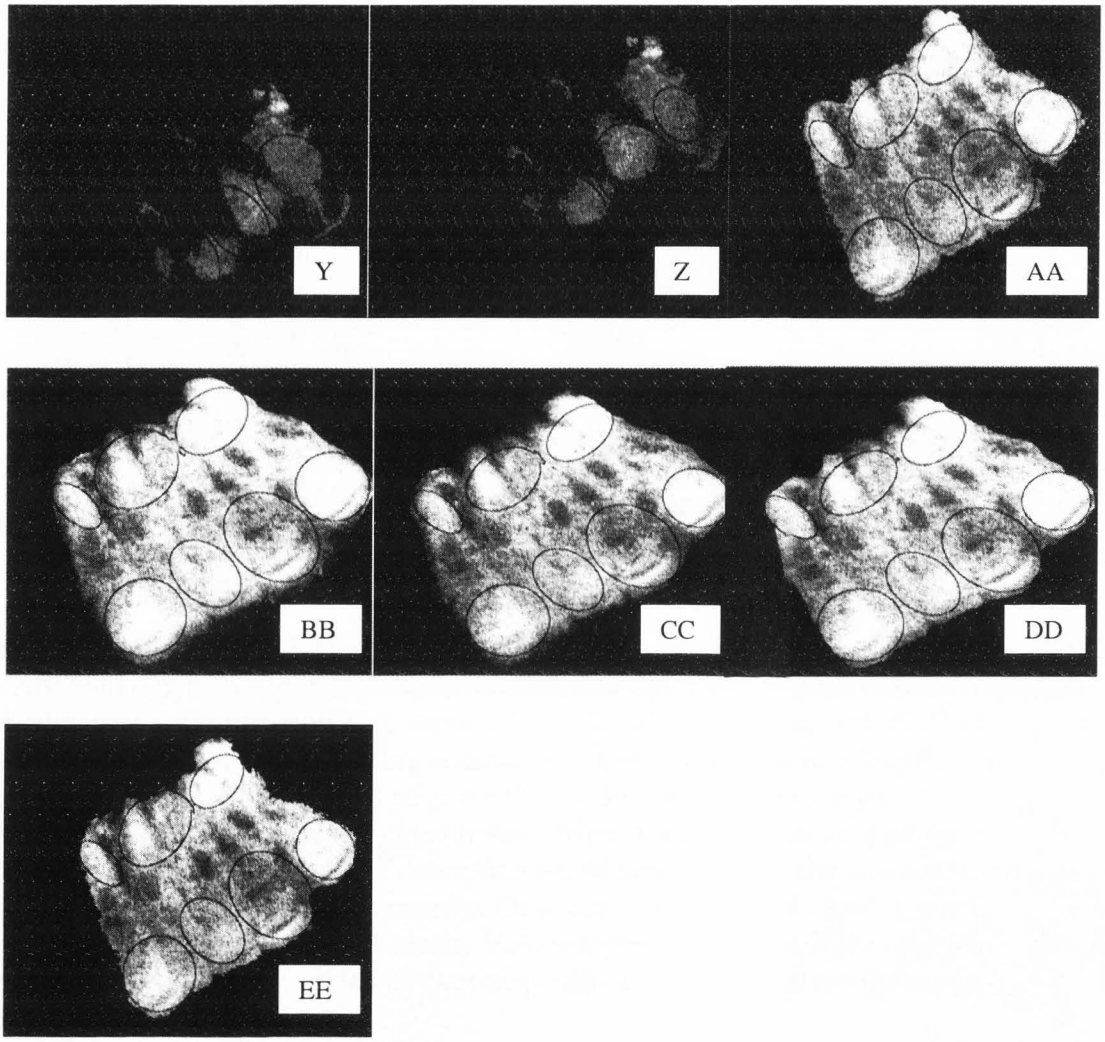
Relative percents of controls were 160%, 211%, and 124% at T=3, 5, and 10 min, respectively.

Pilot studies in the presence and absence of bPTH(1-34) indicated that ^{45}Ca efflux was not affected by either chelerythrine or Rp-cAMP (data not shown).

Effect of bPTH(1-34) on Endocytotic Distribution of FM 1-43

A similar time course was again employed using confocal microscopy. The confocal images of enterocytes exposed to bPTH (1-34) failed to produce the pronounced



fluctuation in fluorescent labeling observed with the steroid hormone $1,25(OH)_2D_3$, but instead resulted in the appearance of large punctate patterns of fluorescence in the apical region of the cells (Figs. 4D-4G) within 3–10 min of hormone. Within the same time period, punctate fluorescence obscured cell nuclei as fluorescent label appeared to translocate from the apical to the basal region of the cells (compare Figs. 4C and 4G).

Effect of $25(OH)D_3$ on Calcium Uptake

Figure 5 demonstrates that 100 nM $25(OH)D_3$ significantly increases ^{45}Ca uptake in isolated cells at 5 and 10 min ($P<0.05$ relative to corresponding controls). The

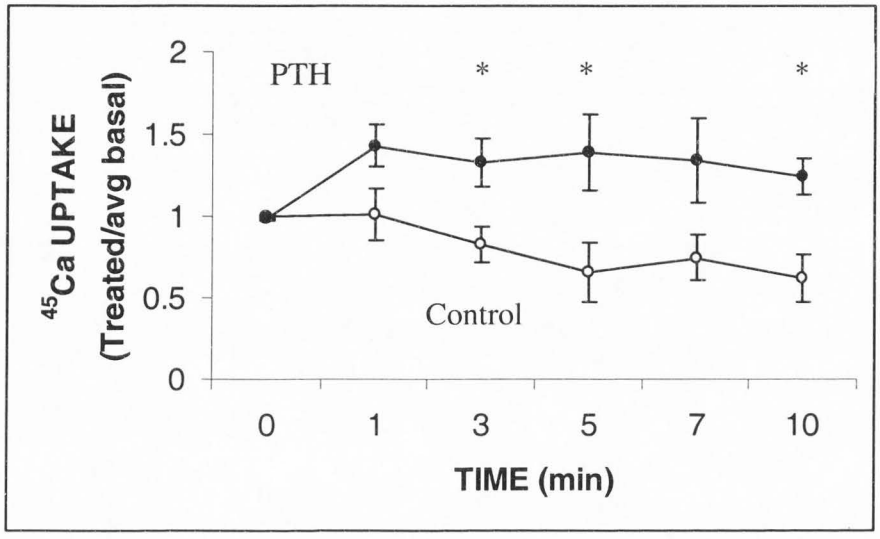


Fig. 3. Time course of bPTH(1-34)-mediated ^{45}Ca uptake in isolated intestinal epithelial cells. Enterocytes were isolated and incubated as described in Fig. 1. The incubation media were carefully aspirated, replaced with RPMI containing 10% FBS and incubated for an additional 24 h. Following addition of $^{45}CaCl_2$ and a 10 min basal incubation period, cells were treated with 65 pM bPTH(1-34) (●–●) or as controls (○–○) and washed with GBSS at the indicated times. After lyses in buffer containing 0.1% Triton X-100, aliquots were taken for determination of protein using the Bradford dye, and for liquid scintillation spectrophotometry. Data were calculated as cpm/μg protein and then related to corresponding basal levels. Values represent mean ± SEM (n = 6). Significant differences ($P<0.05$) are relative to corresponding controls and are indicated by an asterisk (*).

treated/avg basal levels were 1.25 ± 0.11 and 1.54 ± 0.12, respectively, with 162% and 175% of control values at 5 and 10 min of treatment. There was a moderate and consistent increase in treated/avg basal levels at T=1, 3, 5, and 7 min when compared to

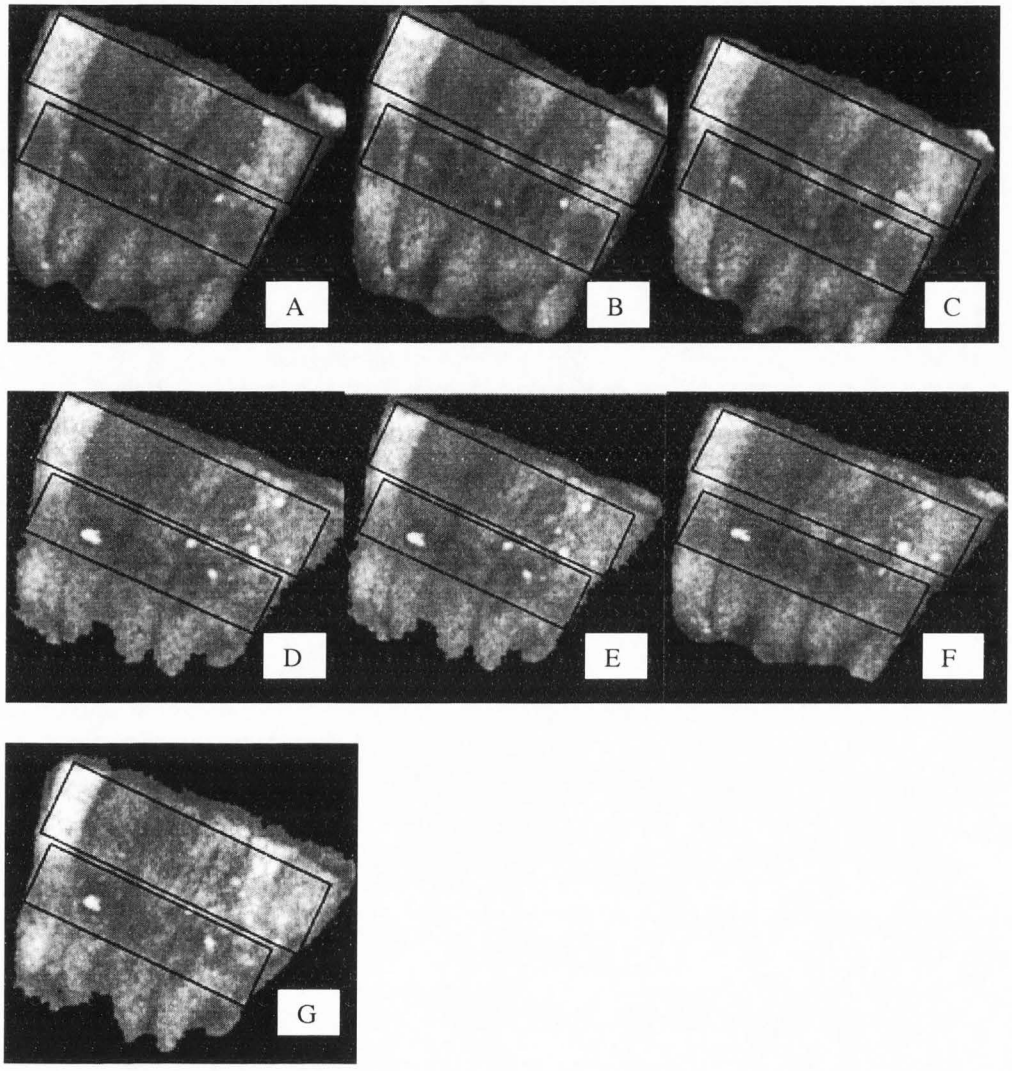


Fig. 4. Effect of bPTH(1-34) on endocytotic distribution of FM 1-43. Micrographs are representative of three independent experiments. Cells were plated overnight in chambered coverslips and for an additional 24 h with 10% FBS. For microscopy, media were aspirated and replaced with GBSS containing dye, which was then overlayed onto the cells. After incubation with the dye, GBSS containing hormone was added to the media and images were captured and analyzed as described in Fig. 2. (A) control; (B) add hormone; (C) end hormone; (D) 1 min; (E) 3 min; (F) 5 min; (G) 9 min.

controls, followed by a dramatic rise in uptake after 10 min. In contrast, intestinal epithelial cells in suspension demonstrated decreased levels of ^{45}Ca in $25(OH)D_3$ –treated cells, relative to controls. [29]

Effect of $25(OH)D_3$ on Endocytotic Distribution of FM 1-43

In confocal studies, intense changes in fluorescent patterns were not detected after treatment with $25(OH)D_3$ (Fig. 6). However, a slight increase in apical fluorescence was observed after 9 min (Fig. 6F).

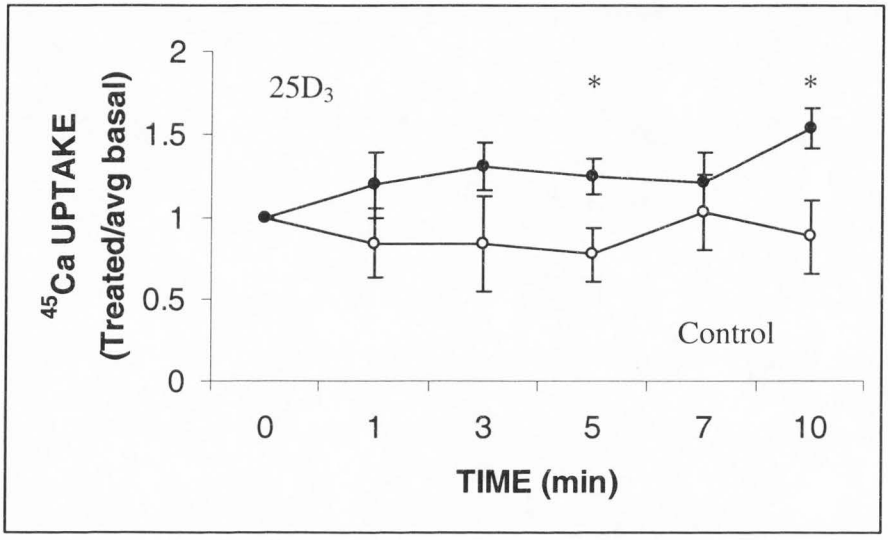


Fig. 5. Time course of $25(OH)D_3$-mediated ^{45}Ca uptake in isolated intestinal epithelial cells. Enterocytes were isolated, incubated and analyzed as described in Figure 1. Cells were treated with 100 nM $25(OH)D_3$ (●–●) or vehicle (0.01% ethanol, final concentration, ○–○), and washed with GBSS at the indicated times. Data were calculated as cpm/μg protein and then related to corresponding basal levels. Values represent mean ± SEM (n=7). Significant differences ($P<0.05$) are relative to corresponding controls and are indicated by an asterisk (*).

Effects of Signal Transduction Inhibitors on 1,25(OH)$_2$D$_3$-Mediated Phosphate Uptake

Figures 7A and 7B illustrate the effects of chelerythrine (a PKC inhibitor) and Rp-cAMP (a PKA inhibitor) on phosphate uptake. When enterocytes were treated with either chelerythrine (Fig. 7A) or Rp-cAMP (Fig. 7B) in the presence of 1,25(OH)$_2$D$_3$ and compared with 1,25(OH)$_2$D$_3$-mediated ^{32}P uptake, no decreases in radionuclide levels were observed. However, enhanced ^{32}P uptake was evident in cells treated with 1,25(OH)$_2$D$_3$ and Rp-cAMP as early as 1 min relative to cells treated with 1,25(OH)$_2$D$_3$

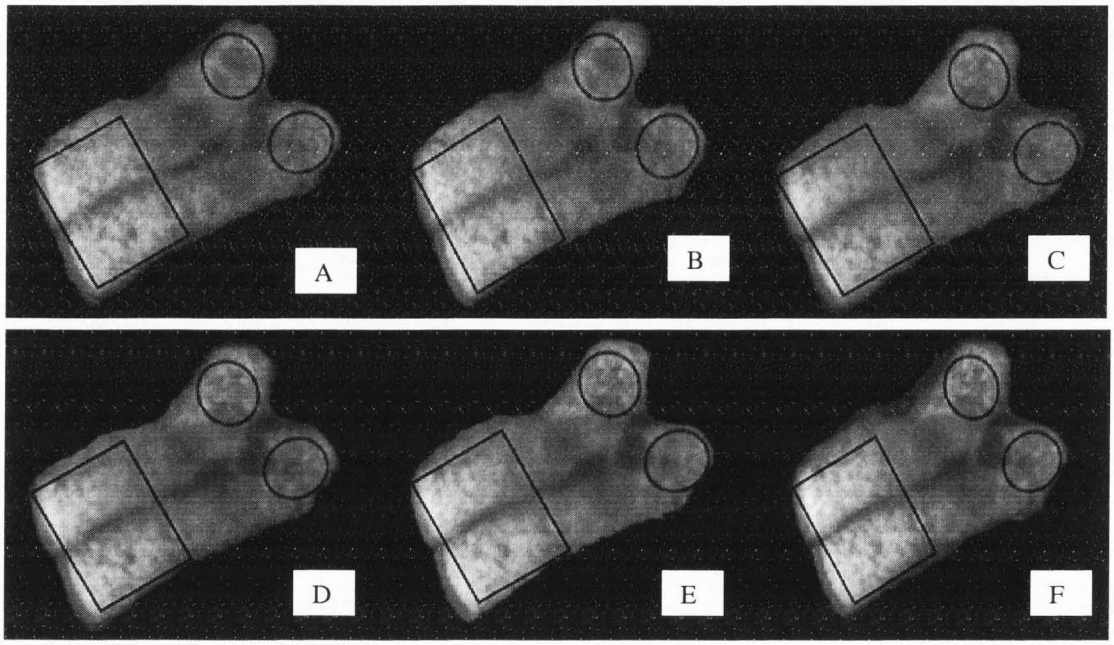


Fig. 6. Effect of 25(OH)D$_3$ on endocytotic distribution of FM 1-43 in cultured intestinal epithelial cells. Confocal images of cells labeled with FM1-43 then treated with 25(OH)D$_3$. Micrographs are representative of three independent experiments. Cells were plated overnight in chambered coverslips. The dye was diluted into GBSS, which was then overlayed onto the cells. After incubation with the dye, GBSS containing hormone was added to the medium and images were captured and analyzed as described in Fig. 2. (A) control; (B) add hormone; (C) 3 min; (D) 5 min; (E) 7 min; (F) 9 min.

only (Fig. 7B) and was significantly higher at 3 min ($P<0.05$), relative to corresponding value for steroid alone.

These results prompted an investigation into the effects of the inhibitors on ^{32}P efflux from cells. In these studies, four conditions were monitored: controls, cells pretreated with inhibitor, $1,25(OH)_2D_3$-treated cells, and $1,25(OH)_2D_3$-treated cells pretreated with inhibitor.

After incubation without or with inhibitor, cells were labeled for 7 min with ^{32}P, then washed twice, and overlayed with 3 ml GBSS in the absence of radionuclide. After removal of an aliquot of medium, cells were treated with vehicle (0.01% ethanol, final concentration) or hormone and additional samples removed at 1, 3, 5, 7, and 10 min.

As shown in Figure 8, chelerythrine pretreatment resulted in an inhibition in ^{32}P efflux in controls (approximately 40%, Fig. 8A) and $1,25(OH)_2D_3$-treated cells (approximately 30%, Fig. 8B). Hormone alone did not alter efflux from control values (Figs. 8A and 8B).

Similar efflux studies following pre-treatment with Rp-cAMP failed to reveal any effect of this PKA antagonist on ^{32}P efflux in the absence or presence of $1,25(OH)_2D_3$ (data not shown).

Effect of Ab099 on $1,25(OH)_2D_3$ –Mediated ^{32}P Uptake

To assess the role of a putative membrane receptor $1,25(OH)_2D_3$ MARRS (**m**embrane **a**ssociated, **r**apid **r**esponse **s**teroid-binding) protein, on phosphate uptake, cultured cells were preincubated in the absence and presence of Ab099. Radionuclide and vehicle or hormone were then added and the incubation continued an additional 7

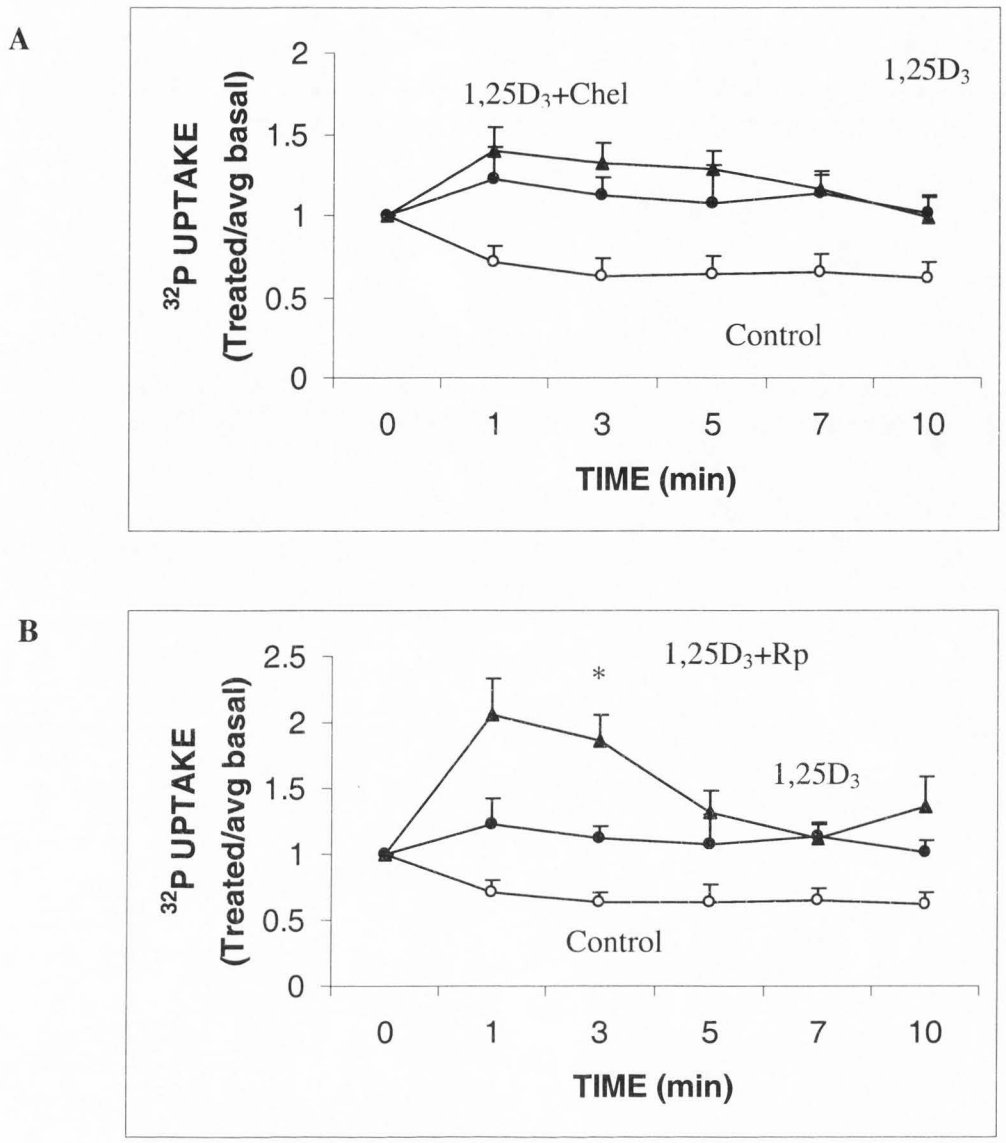
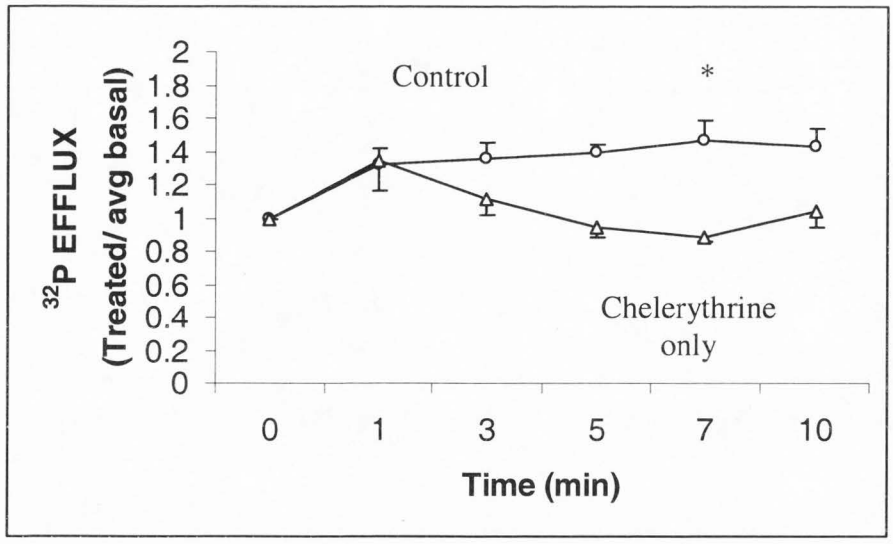


Fig. 7. (A) Effect of the PKC inhibitor, chelerythrine (n=9), or (B) the PKA inhibitor, Rp-cAMP (n=6), on 1,25(OH)D_3-mediated ^{32}P uptake in isolated intestinal cells. Enterocytes were isolated, incubated and analyzed as described in Fig. 1. Cells were treated with inhibitor and 1,25$(OH)_2D_3$ (▲−▲), 1,25$(OH)_2D_3$ only (●−●), or vehicle (0.01% ethanol, ○−○). Data were calculated as cpm/μg protein and then related to corresponding basal levels, values ± SEM. Significant differences ($P<0.05$) are compared between inhibitor and 1,25$(OH)_2D_3$ or 1,25$(OH)_2D_3$ only and are denoted by and asterisk (*).

A



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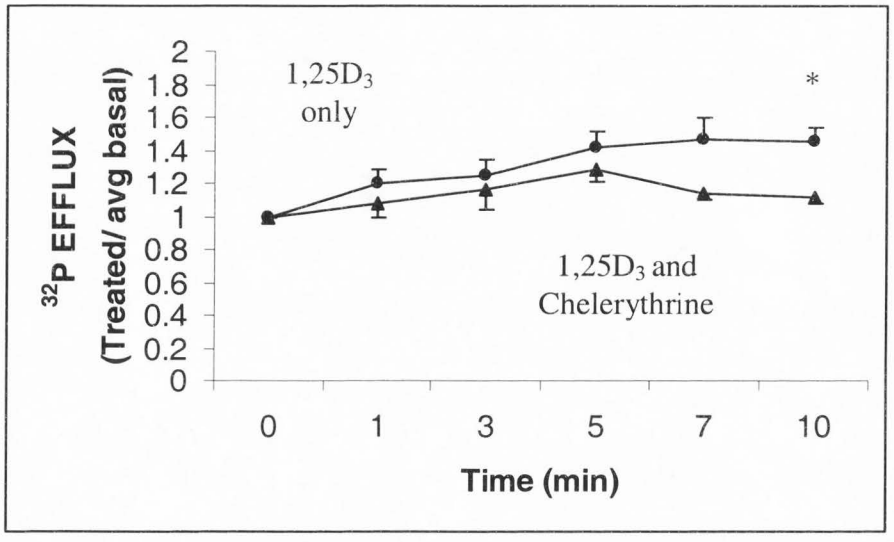


Fig. 8. Effect of chelerythrine on ^{32}P efflux from isolated chick enterocytes. (A) ^{32}P efflux from cells (○–○) and controls pretreated with 0.66μM chelerythrine for 3 h (Δ–Δ) (n=3). (B) ^{32}P efflux from 1,25(OH)$_3$D$_3$-treated cells (●–●) and cells pretreated with chelerythrine followed by hormone (▲–▲) (n=3). Intestinal cells were isolated and incubated as described in Fig. 1. After overnight incubation, cells were treated with 0.66 μM chelerythrine for 3 h prior to experimentation. Cells were labeled with ^{32}P for 7 min, washed, and placed in GBSS lacking radionuclide. One hundred-μl aliquots of media were removed at the indicated times. Data were calculated as cpm and then related to corresponding basal values. Significant difference ($P<0.05$) is compared between control and 1,25(OH)$_3$D$_3$ values with or without chelerythrine and is denoted by an asterisk (*).

min. Figure 9 illustrates that 130 pM $1,25(OH)_2D_3$ increased ^{32}P uptake to 170% of controls. Preincubation of cells with Ab099 showed no significant difference in ^{32}P uptake when compared to controls but completely inhibited hormone stimulated uptake.

Effect of Inhibitors and $1,25(OH)_2D_3$-Mediated Changes on Endocytotic Distribution of FM 1-43

Pretreatment of cells with chelerythrine effectively blocked the rapid $1,25(OH)_2D_3$-mediated changes in FM 1-43 fluorescence (data not shown). Instead, a gradual increase in cellular fluorescence became noticeable at the basal region of the cells

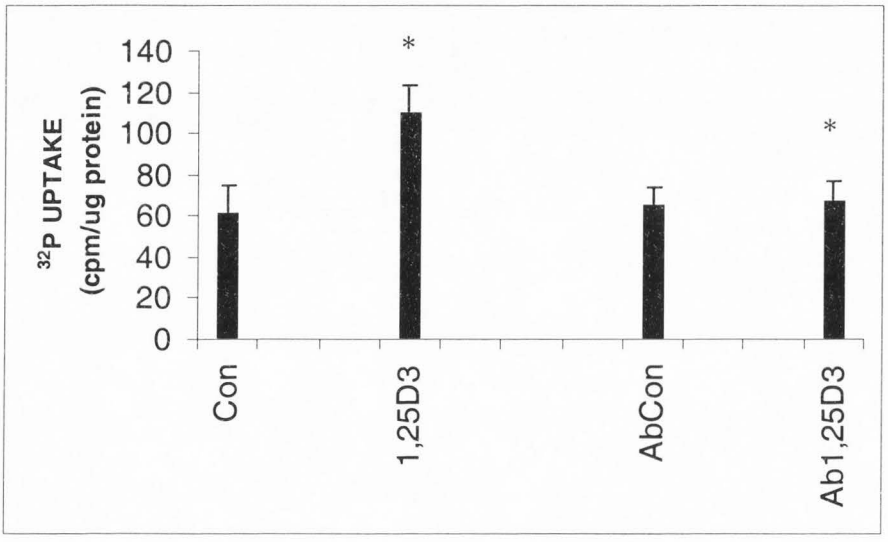


Fig. 9. Effect of Ab099 on ^{32}P uptake in isolated intestinal epithelial cells. Conditions for isolation, incubation, and analysis of enterocytes were as described in Fig. 1. After overnight culture, cells were cultured with GBSS-0.1% BSA without or with Ab099 (1/500 dilution) for 5 min, then exposed to GBSS containing ^{32}P, and either vehicle (0.01% ethanol) or $1,25(OH)_3D_3$ and incubated for 7 min. Data were calculated as cpm/μg protein and then related to corresponding basal levels. Values represent mean ± SEM. Significant difference ($P<0.05$) is compared between Ab099 and $1,25(OH)_3D_3$ or $1,25(OH)_2D_3$ only and is denoted by and asterisk (*).[47]

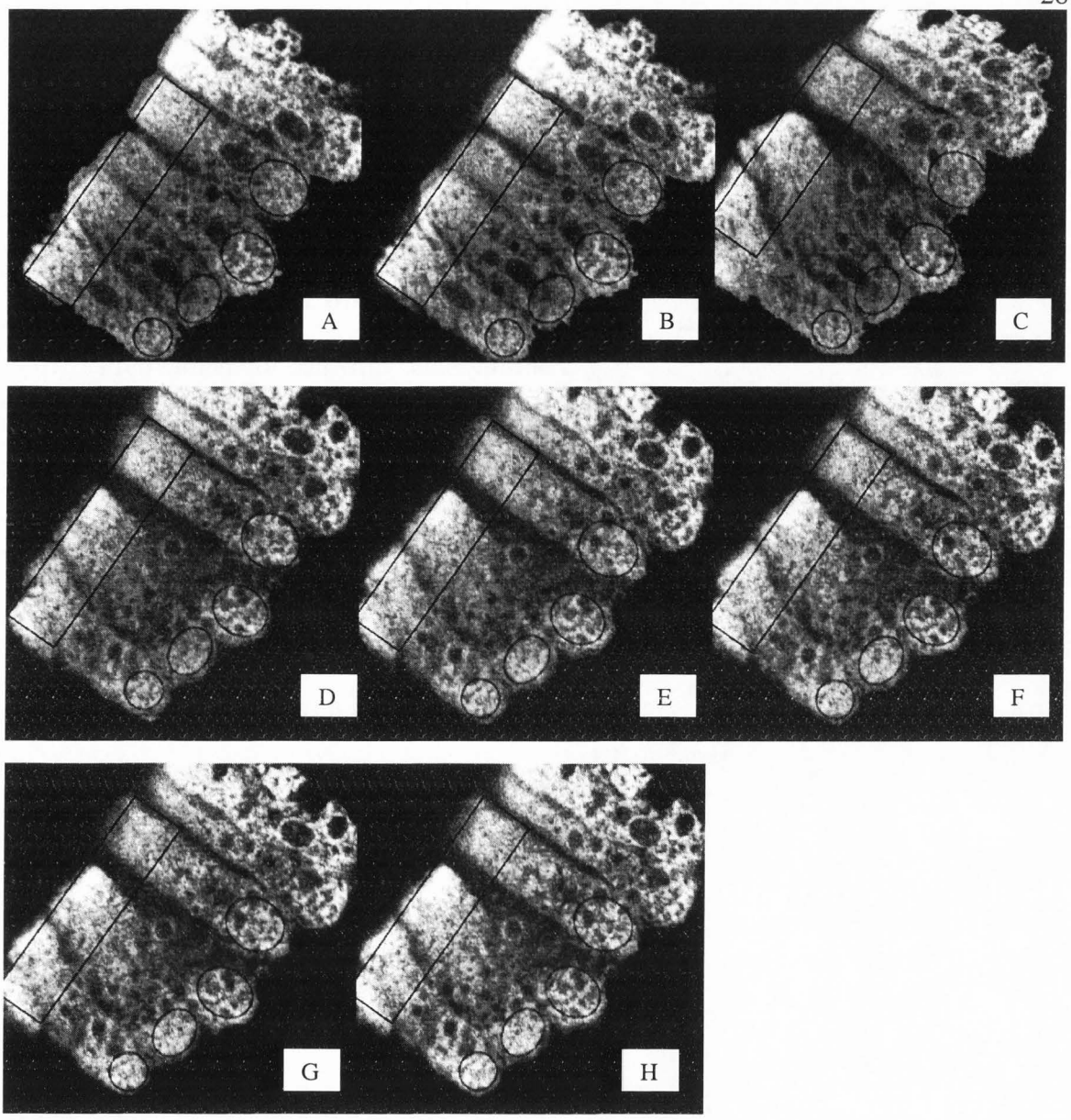


Fig. 10. Effect of chelerythrine and $1,25(OH)_2D_3$ on endocytotic distribution of FM 1-43 labeling. Confocal images of cells preincubated with a PKC inhibitor (chelerythrine), labeled with FM1-43, then treated with $1,25(OH)_2D_3$. Micrographs are representative of two independent experiments. Cells were plated overnight in chambered coverslips. The dye was diluted into GBSS, which was then overlayed onto the cells. After incubation with the dye, medium was replaced with GBSS containing hormone and images were captured and analyzed as described in Fig. 2. (A) control; (B) add hormone; (C) 10 sec; (D) 1 min; (E) 3 min; (F) 5 min; (G) 7 min; (H) 9 min.

after 1 min (compare Figs. 10A and 10D), which increased over time (Figs. 10E-10H). In some cells, an increase in apical fluorescence was also noted (compare Figs. 10A and 10H).

Effect of Inhibitors and 1,25$(OH)_2D_3$-Mediated Changes on Endocytotic Distribution of FM 1-43

Pretreatment of cell with chelerythrine effectively blocked the rapid 1,25$(OH)_2D_3$-mediated changes in FM 1-43 fluorescence (data not shown). Instead, a gradual increase in cellular fluorescence became noticeable at the basal region of the cells after 1 min (compare Figs. 10A and 10D), which increased over time (Figs. 10E-10H). In some cells, an increase in apical fluorescence was also noted (compare Figs. 10A and 10H).

Cells pretreated with Rp-cAMP (Fig. 11) exhibited an increase in apical fluorescence within 10 sec to 1 min of hormone (Figs. 11C and 11D). Fluorescence intensity increased dramatically as time progressed (Figs. 11E-11H). Again the very rapid steroid-mediated changes in fluorescence were absent (data not shown).

Preincubation of cells with Ab099 also abolished rapid 1,25$(OH)_2D_3$-mediated changes in FM 1-43 distribution (data not shown), but as indicated in Fig. 12 some relocalization of dye occurred as judged by the disappearance of nuclear outlines in the two cells depicted between 1-9 min after hormone (Figs. 12D-12H).

Effects of Signal Transduction Inhibitors on bPTH(1-34)-Mediated Calcium Uptake

The potential involvement of PKA and PKC pathways in PTH-mediated ^{45}Ca uptake was investigated. Cells were treated with inhibitor and bPTH(1-34), bPTH(1-34) only or vehicle. When exposed to both the PKC inhibitor, chelerythrine, and bPTH(1-

34), no inhibition of ^{45}Ca uptake was observed (Fig. 13A). However, inhibition was evident in cells treated with PKA inhibitor, Rp-cAMP, in the presence of bPTH(1-34). Fig. 13B shows that the presence of Rp-cAMP caused significant decreases in ^{45}Ca at T=1, 3, and 10 min ($P<0.05$, relative to corresponding levels for hormone alone). When

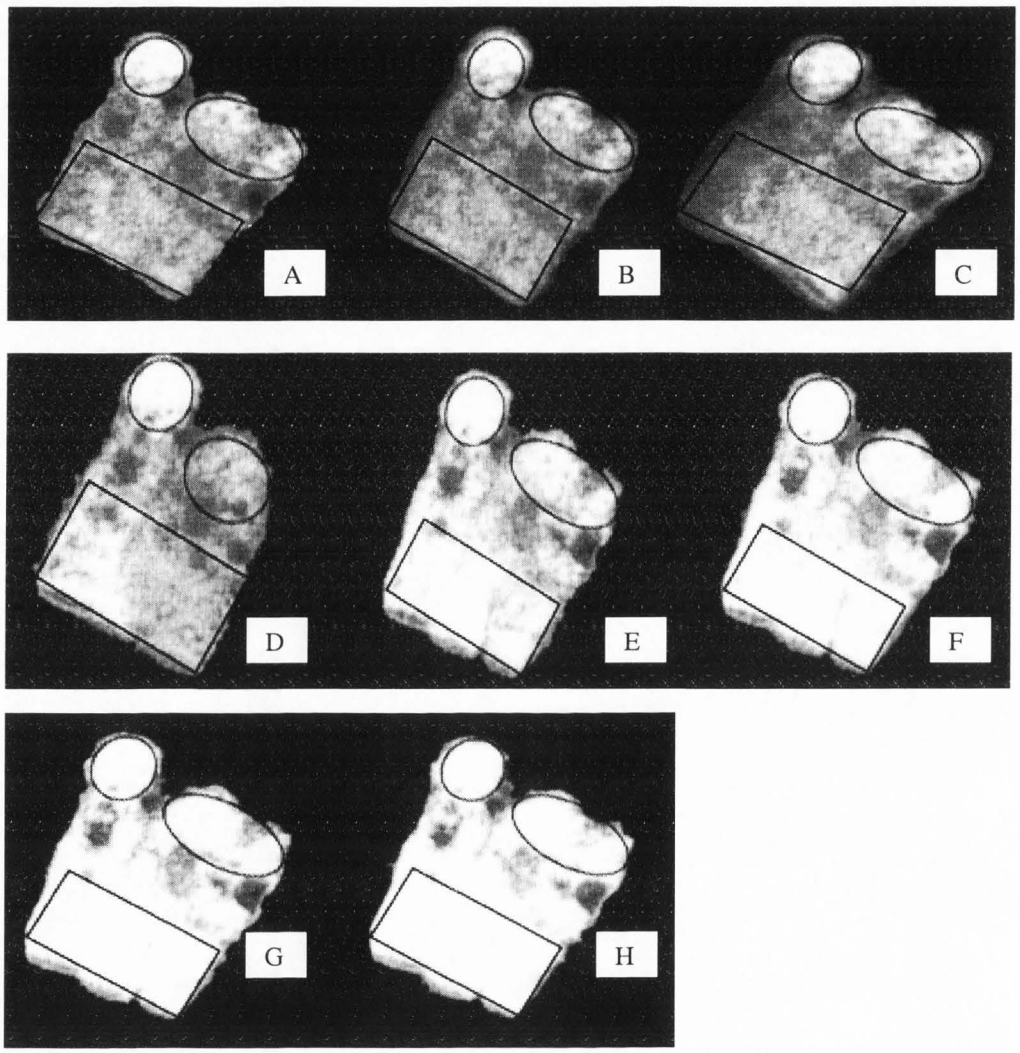


Fig. 11. Effect of Rp-cAMP and $1,25(OH)_2D_3$ on endocytotic distribution of FM 1-43 labeling. Confocal images of cells preincubated with a PKA inhibitor (Rp-cAMP), labeled with FM1-43, then treated with $1,25(OH)_2D_3$. Micrographs are representative of two independent experiments. Cells were plated overnight in chambered coverslips. The dye was diluted into GBSS, which was then overlayed onto the cells. After incubation with the dye, medium was replaced with GBSS containing hormone and images were captured and analyzed as described in Fig. 2. (A) control; (B) add hormone; (C) 10 sec; (D) 1 min; (E) 3 min; (F) 5 min; (G) 7 min; (H) 9 min.

compared to cells with bPTH(1-34) alone, the observed levels in Rp-cAMP-treated cells fell below 100% to 70%, 71% and 74% at 1, 3, and 10 min, respectively.

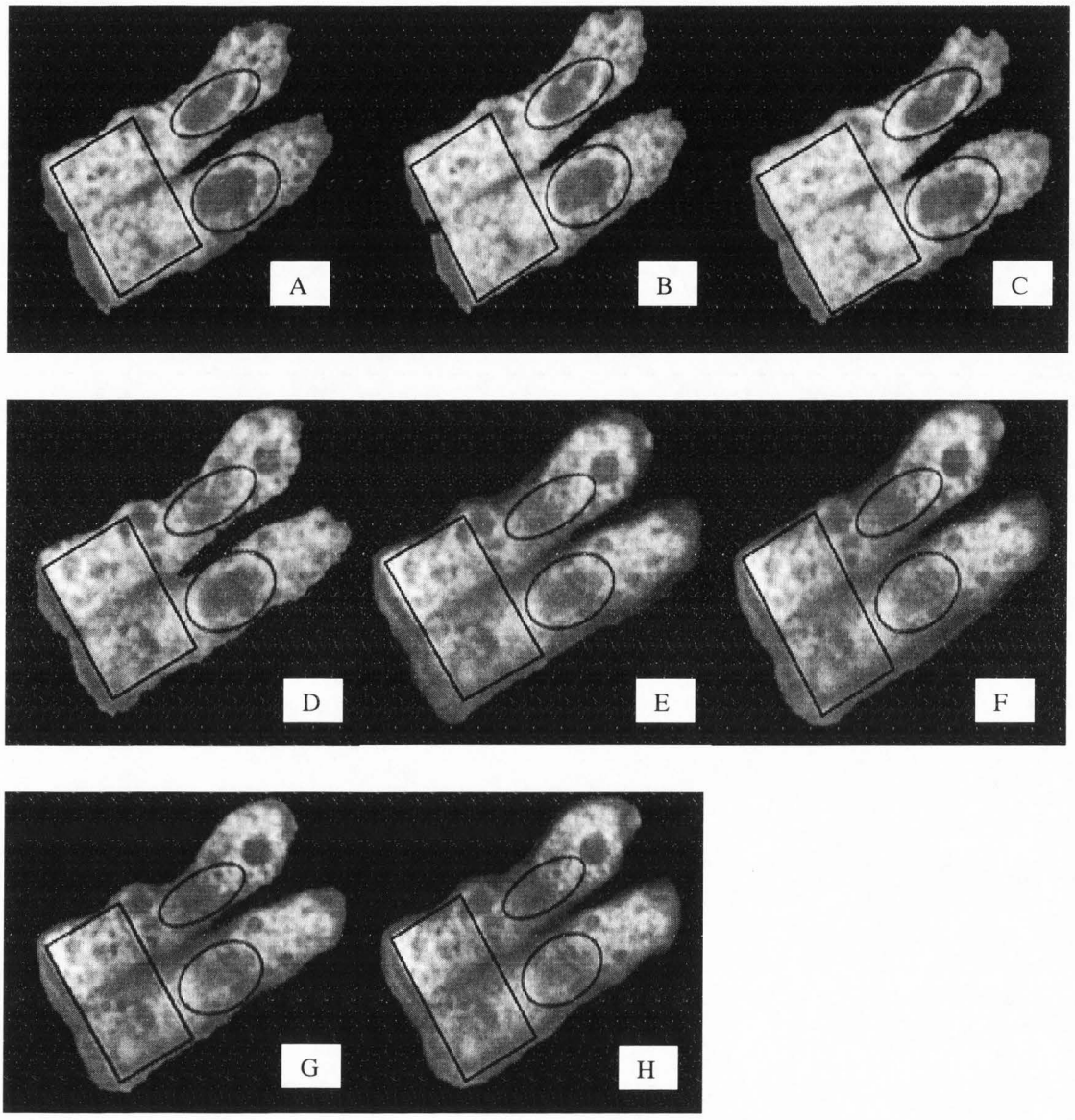


Fig. 12. Effect of Ab099 and $1,25(OH)_2D_3$ on endocytotic distribution of FM 1-43 labeling. Confocal images of cells preincubated with Ab099, labeled with FM1-43, then treated with $1,25(OH)_2D_3$. Micrographs are representative of two independent experiments. Cells were plated overnight in chambered coverslips. The dye was diluted into GBSS, which was then overlayed onto the cells. After incubation with the dye, medium was replaced with GBSS containing hormone and images were captured and analyzed as described in Fig. 2. (A) control; (B) add hormone; (C) 10 sec; (D) 1 min; (E) 3 min; (F) 5 min; (G) 7 min; (H) 9 min.

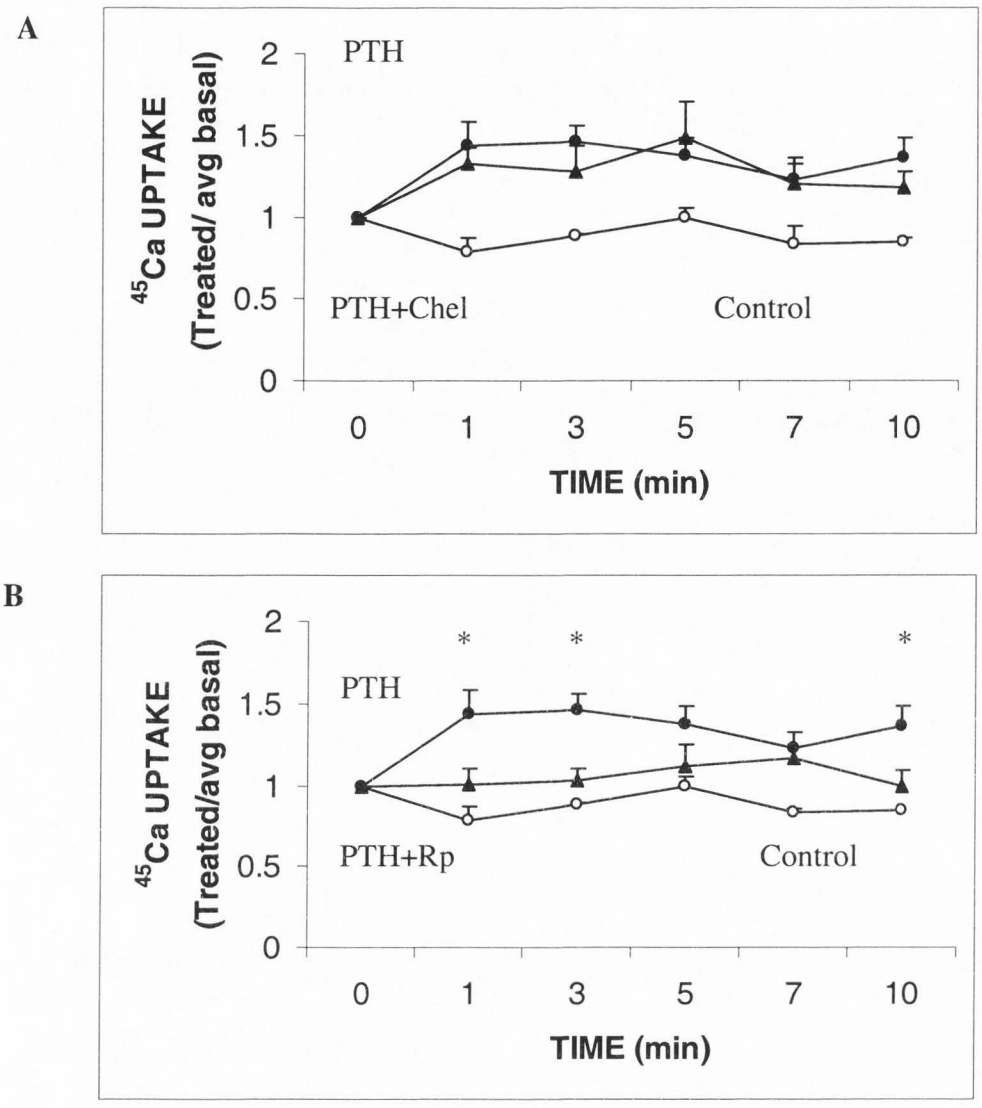


Fig. 13. (A) Effect of PKC inhibitor, chelerythrine (n=6), or (B) PKA inhibitor, Rp-cAMP (n=6), on bPTH (1-34)-mediated ^{45}Ca uptake in isolated intestinal cells. Cells were treated with inhibitor and bPTH (1-34) (▲–▲), bPTH (1-34) only (●–●), or control (○–○). Enterocytes were isolated, incubated and analyzed as described in Figure 6. Data were calculated as cpm/μg protein and then related to corresponding basal levels, values ± SEM. Significant differences ($P<0.05$) are compared between inhibitor and PTH or PTH only and are denoted with an asterisk (*).

Effect of Inhibitors on bPTH(1-34)-Mediated Changes on Endocytotic Distribution of FM 1-43

Figure 14 depicts confocal images of intestinal cells preincubated with chelerythrine (Fig. 14A) followed by addition of bPTH(1-34) (Figs. 14B-14G). Between 10 sec and 1 min of hormone addition, localized apical fluorescence was observed to increase (Figs. 14E and 14F).

Equivalent experiments with Rp-cAMP resulted in a noticeable increase in apical fluorescence, which was particularly noticeable 3 min after hormone addition (Fig. 15D), increasing through 9 min after hormone addition (Fig. 15G). The increase in fluorescence in the apical membrane became sufficiently extensive to obscure in punctate staining.

Effects of Signal Transduction Inhibitors on 25(OH)D_3-Mediated Calcium Uptake

Treatment of isolated intestinal cells with a PKC inhibitor, chelerythrine had no significant effect on 25(OH)D_3-mediated calcium uptake (Figure 16A). On the other hand, a PKA inhibitor, Rp-cAMP, significantly decreased the effect of 25(OH)D_3-mediated calcium uptake at 10 min (Figure 16B).

Effect of Inhibitors on 25(OH)D_3-Mediated Changes on Endocytotic Distribution of FM 1-43

The confocal images in Figures 17 and 18 illustrate enterocytes preincubated with chelerythrine or Rp-cAMP, respectively, exposed to FM1-43 and treated with 25(OH)D_3. Cells preincubated with chelerythrine resulted in no change in fluorescence (Fig. 17). In contrast, cells preincubated with Rp-cAMP demonstrated an overall increase in

fluorescence throughout the length of the cells (Fig. 18), which became more intense with time (Figs. 18B-18F).

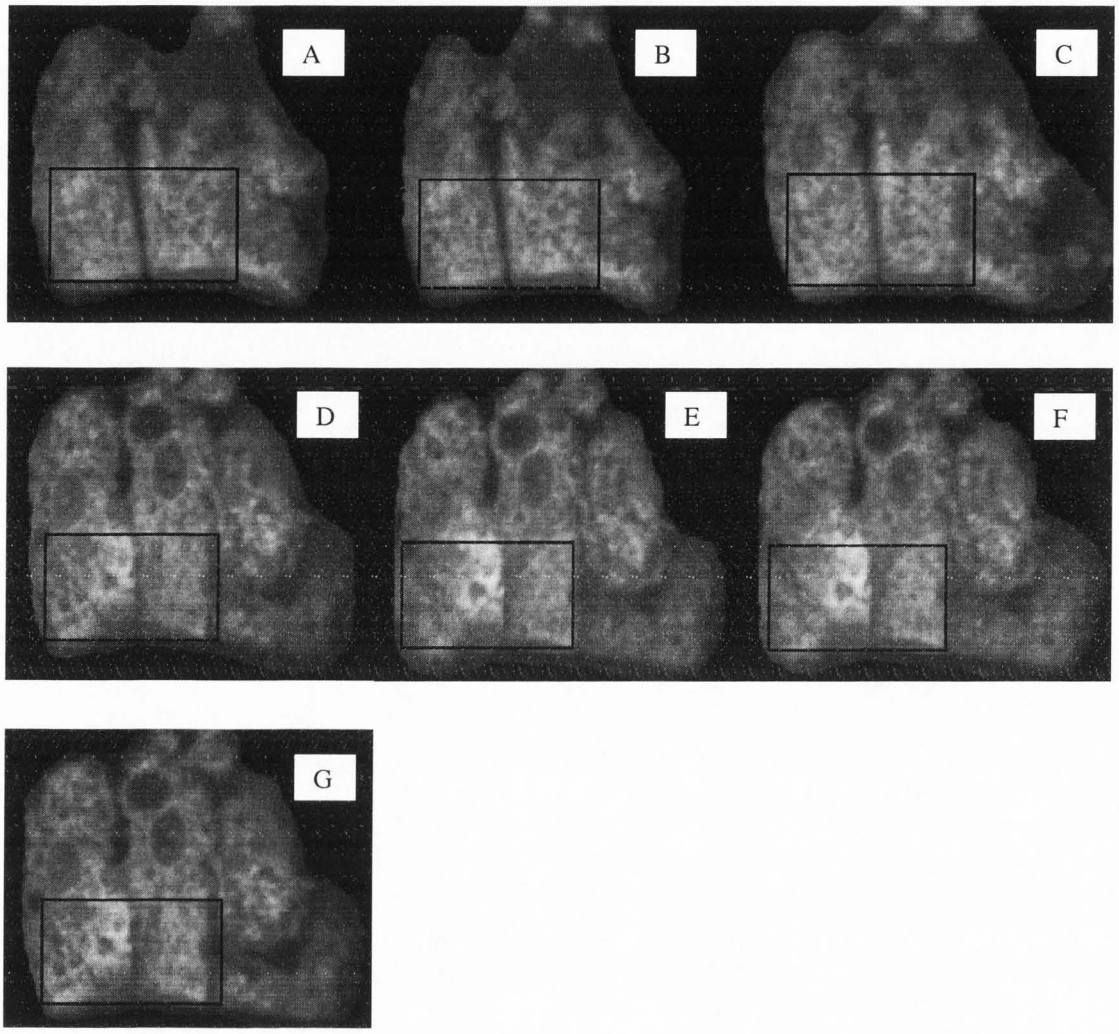


Fig. 14. Effect of chelerythrine and bPTH(1-34) on endocytotic distribution of FM 1-43 labeling. Confocal images of cells preincubated with a PKC inhibitor (chelerythrine), labeled with FM1-43, then treated with bPTH(1-34). Micrographs are representative of two independent experiments. Cells were plated overnight in chambered coverslips. The dye was diluted into GBSS, which was then overlayed onto the cells. After incubation with the dye, GBSS containing hormone was added to the medium and images were captured and analyzed as described in Fig. 2. (A) control; (B) add hormone; (C) end hormone; (D) 1 min; (E) 3 min; (F) 5 min; (G) 9 min.

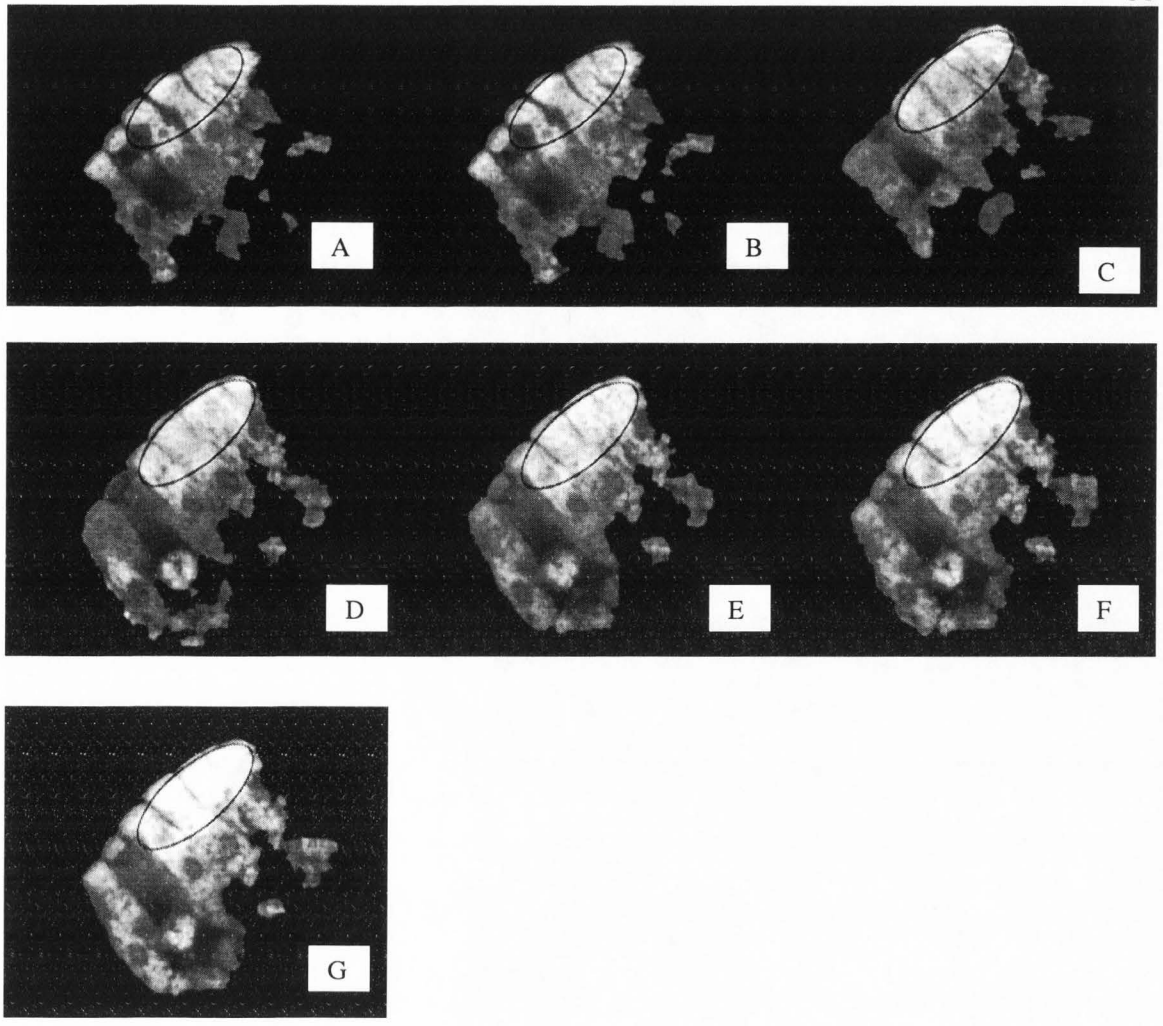
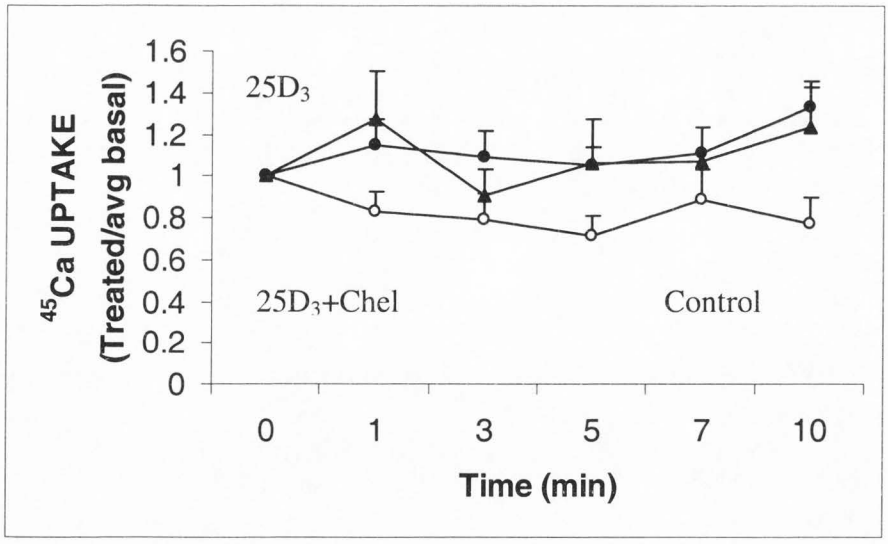


Fig. 15. Effect of Rp-cAMP and bPTH(1-34) on endocytotic distribution of FM 1-43. Confocal images of cells preincubated with a PKA inhibitor (Rp-cAMP), labeled with FM1-43, then treated with bPTH(1-34). Micrographs are representative of two independent experiments. Cells were plated overnight in chambered coverslips. The dye was diluted into GBSS, which was then overlayed onto the cells. After incubation with the dye, GBSS containing hormone was added to the medium and images were captured and analyzed as described in Fig. 2. (A) control; (B) add hormone; (C) end hormone; (D) 1 min; (E) 3 min; (F) 5 min; (G) 9 min.

A



B

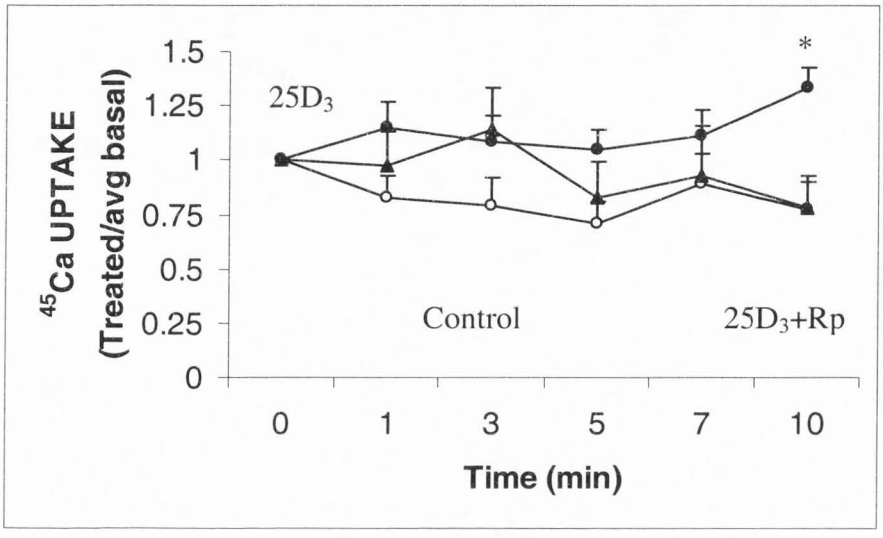


Fig. 16. (A) Effect of PKC inhibitor, chelerythrine (n=7), or (B) PKA inhibitor, Rp-cAMP (n=7), on 25(OH)D$_3$-mediated ^{45}Ca uptake in isolated intestinal cells. Cells were treated with inhibitor and 25(OH)D$_3$(▲–▲), 25(OH)D$_3$ only (●–●), or vehicle (0.01% ethanol, ○–○). After culturing as described in Fig. 1. Data were calculated as cpm/μg protein and then related to corresponding basal levels. Values represent mean ± SEM. Significant difference ($P<0.05$) is compared between inhibitor and 25(OH)D$_3$ or 25(OH)D$_3$ only and is denoted by and asterisk (*).

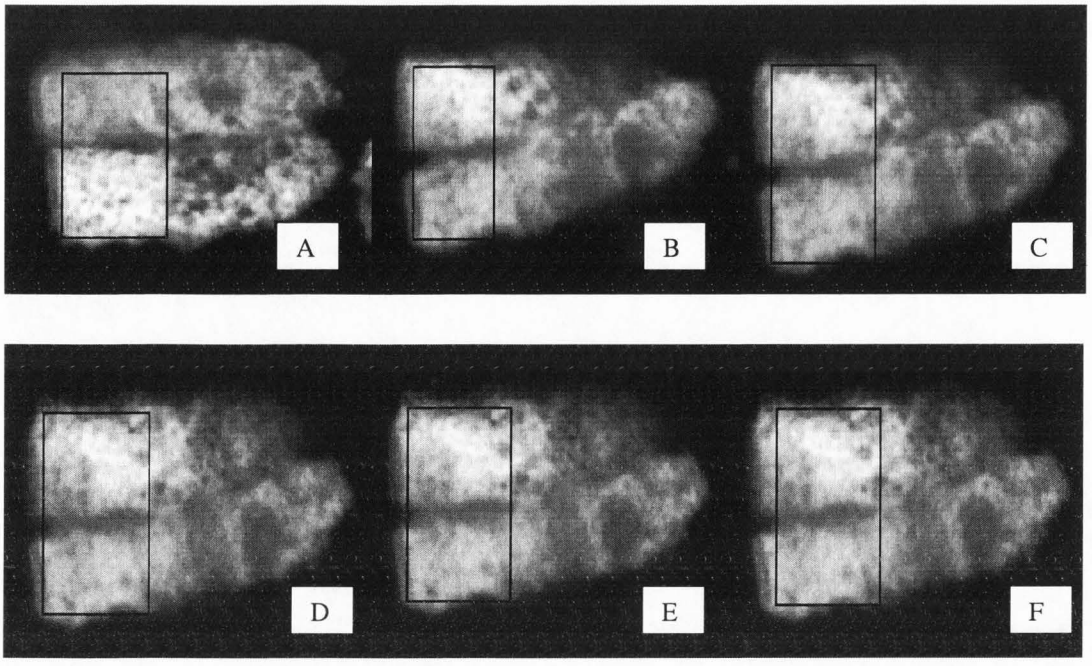


Fig. 17. Effect of chelerythrine and 25(OH)D_3 on endocytotic distribution of FM 1-43. Confocal images of cells preincubated with a PKC inhibitor (chelerythrine), labeled with FM1-43, then treated with 25(OH)D_3. Micrographs are representative of two independent experiments. Cells were plated overnight in chambered coverslips. The dye was diluted into GBSS, which was then overlayed onto the cells. After incubation with the dye, GBSS containing hormone was added to the medium and images were captured and analyzed as described in Fig. 2. (A) control; (B) 1.4 min; (C) 3 min; (D) 5 min; (E) 7 min; (F) 9 min.

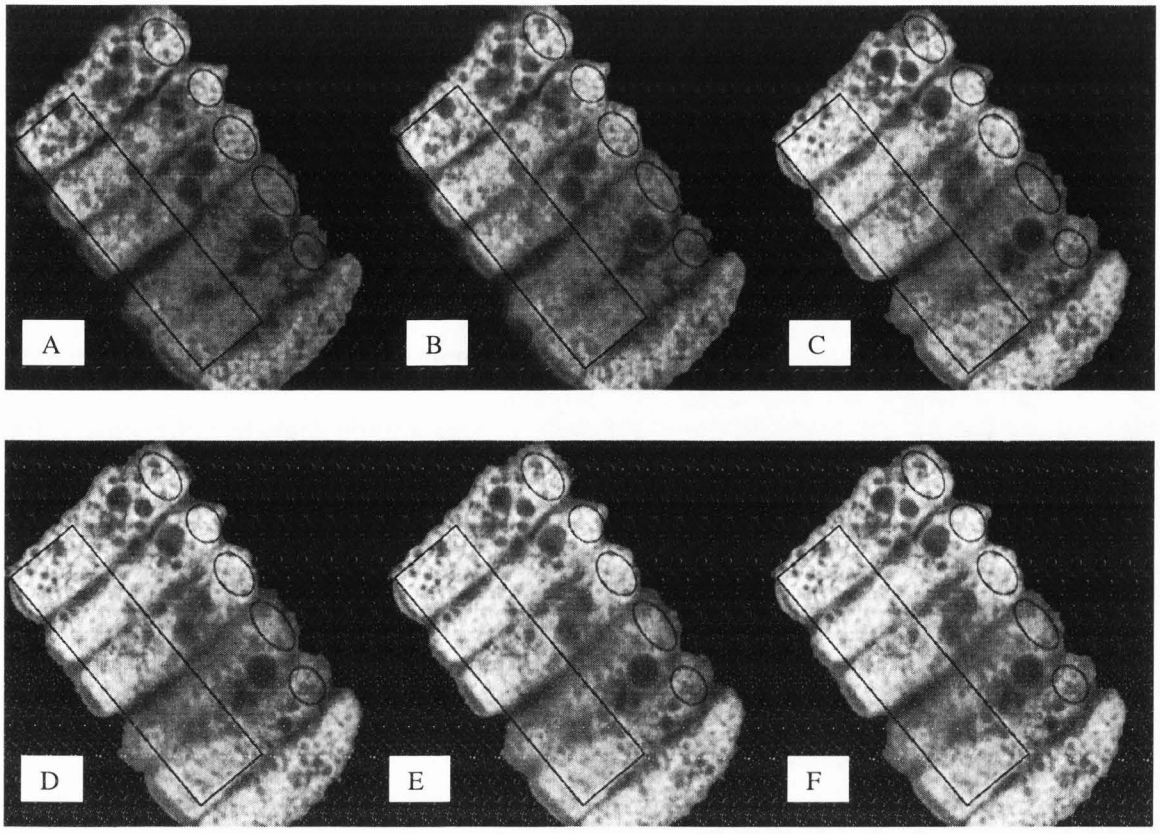


Fig. 18. Effect of Rp-cAMP and 25(OH)D_3 on endocytotic distribution of FM 1-43. Confocal images of cells preincubated with a PKA inhibitor (Rp-cAMP), labeled with FM1-43 and then treated with 25(OH)D_3. Micrographs are representative of two independent experiments. Cells were plated overnight in chambered coverslips. The dye was diluted into GBSS, which was then overlayed onto the cells. After incubation with the dye, GBSS containing hormone was added to the medium and images were captured and analyzed as described in Fig. 2. (A) control; (B) add hormone; (C) 3 min; (D) 5 min; (E) 7 min; (F) 9 min.

CHAPTER V

DISCUSSION

1,25-Dihydroxyvitamin D_3

Previous experiments with isolated chick enterocytes in suspension have demonstrated that $1,25(OH)_2D_3$ increases phosphate uptake as early as 5 min.[35] In this study with cultured chick enterocytes, an increase in $1,25(OH)_2D_3$-mediated phosphate uptake occurs as early as 1 min and is significantly higher than controls at 3 min. Although the treated/ average basal values decrease in both hormone-treated cells and controls with time compared to basal, these levels are sustained more closely to basal values compared to the continued declines reported with cells in suspension. Perhaps as a consequence, the increase in ^{32}P uptake is sustained at an average of 208% above controls from 3 to 10 min (levels higher than the maximum 151% of control observed at 10 min with cells in suspension).

To identify the role of vesicular transport in the $1,25(OH)_2D_3$-mediated phosphate uptake observed in these cultured enterocytes, cells were cultured on coverslips and an equivalent time course was used in studies with confocal microscopy with FM 1-43. The extremely rapid decrease in apical fluorescence that is observed within 10 sec of $1,25(OH)_2D_3$ suggests an endocytotic mechanism that is consistent with the findings of Warner and Coleman.[4] Their work followed $1,25(OH)_2D_3$-mediated calcium uptake using electron probe analysis and suggested calcium may be endocytosed just below the brush border, appearing as discreet localizations in the apical region, followed by extrusion at the lateral membrane.

Vesicular phosphate transport accumulation has also been proposed to occur on the basis of localization studies in human fibroblast lysosomes[6,27] and lysosomal fractions following perfusion of chick duodenal loops.[5]

To determine the signal transduction events that may facilitate this vectorial transport of phosphate ions, Ab099, in addition to PKC and PKA inhibitors, were employed in uptake experiments for the current study. Likewise, a lysosomal localization of calcium has been reported in chick enterocytes[20] and fibroblasts. [6]

Upon identification of a vitamin D receptor located in the baso-lateral membrane in chick intestine, [48] Nemere et al.[49] isolated a highly specific polyclonal antibody (Ab099) against a synthetic peptide corresponding to the N-terminus of the protein. Using this antibody, Nemere et al. [50] have studied a putative plasmalemmal receptor $1,25(OH)_2D_3$ MARRS protein that initiates $1,25(OH)_2D_3$-mediated phosphate and/or calcium uptake in intestinal cells. In addition to their findings, several studies have demonstrated that $1,25(OH)_2D_3$ MARRS protein is responsible for binding $1,25(OH)_2D_3$ in the baso-lateral membrane.[47,50] This protein has been shown to facilitate the hormone's rapid effects on phosphate and calcium uptake in perfusion studies with chick duodena and in isolated chick enterocytes in suspension.[35,41,51] In the current work, preincubation of isolated intestinal epithelial cells with Ab099 completely inhibits $1,25(OH)_2D_3$-mediated phosphate uptake. These results are also supported by equivalent conditions using confocal microscopy, that show no overall increase in fluorescence nor redistribution of perinuclear fluorescence. In essence, pretreatment of cells with Ab099 inhibits $1,25(OH)_2D_3$-induced changes.

When cultured cells are treated with a PKC inhibitor (chelerythrine) and exposed to $1,25(OH)_2D_3$, there is a modest increase in phosphate uptake. However, cells treated with both a PKA inhibitor (Rp-cAMP) and $1,25(OH)_2D_3$ show elevated phosphate uptake levels to 366% of controls after 3 min, when compared to cells treated only with $1,25(OH)_2D_3$, that exhibited an increase to 202% of controls. These results are validated by confocal microscopy where only slight increases in fluorescent intensity are observed in cells pretreated with chelerythrine, but dramatic increases in fluorescent intensity are observed in cells pretreated with Rp-cAMP. In contrast to these findings, Zhao and Nemere[35] showed that cells in suspension mimicked the $1,25(OH)_2D_3$-mediated increase in phosphate uptake when treated with a PKC activator, whereas those treated with a PKA activator did not. Consequently, additional experiments involving efflux studies were employed to further investigate the complex effects of the PKC and PKA inhibitors in the presence of $1,25(OH)_2D_3$.

In efflux studies, pretreatment of adherent cells with chelerythrine was found to inhibit efflux in cells treated or untreated with $1,25(OH)_2D_3$ at the later time points tested, which may account for the elevated levels of ^{32}P. However, chelerythrine-induced increases in ^{32}P at 1 and 3-min after hormone may be indicative of crosstalk between signaling pathways. Pilot studies with cells preincubated with Rp-cAMP illustrate no apparent change in ^{32}P efflux.

In conclusion, this work with adherent cells demonstrated that $1,25(OH)_2D_3$-mediated phosphate uptake occurs more rapidly (3 min) than cells in suspension (5 min). After exposure to PKC inhibitor (chelerythrine) with or without hormone, results suggest that the PKC signal transduction pathway is involved in the extrusion of phosphate from

the cells. In the presence of $1,25(OH)_2D_3$, the PKA inhibitor (Rp-cAMP) may down-regulate other pathways involved in $1,25(OH)_2D_3$-mediated phosphate uptake. The changes in fluorescent intensity observed during confocal imaging supports the hypothesis that vesicular transport is sufficiently rapid enough to be involved in $1,25(OH)_2D_3$-mediated phosphate uptake. And finally, the abolished effect of $1,25(OH)_2D_3$-mediated phosphate uptake in the presence of Ab099 suggests that the $1,25(OH)_2D_3$-MARRS protein facilitates $1,25(OH)_2D_3$-mediated phosphate uptake in isolated chick enterocytes.

Parathyroid Hormone

Earlier studies in perfusion experiments have demonstrated that bPTH(1-34) increases calcium uptake in perfused chick duodena.[36,38] The current work shows that bPTH(1-34) increases calcium uptake in isolated intestinal epithelial cells cultured in serum. A similar permissive effect of serum for PTH action in cultured rat calvaria was subsequently pinpointed to a requirement for $1,25(OH)_2D_3$ in serum-free conditions.[52] Confocal imaging of an equivalent time course reveals increases in punctate fluorescence that coincides with elevated calcium levels observed at 3, 5, and 10 min. Upon preincubation with chelerythrine and subsequent exposure to bPTH(1-34), no significant changes in calcium uptake were observed although there was a slight increase when compared to hormone only. Confocal images over this time period showed a smaller increase in fluorescence at the apical portion of the cells. It has previously been reported that bPTH(1-34) does not activate PKC within a 10-min time course.[36] Therefore, the slight changes due to chelerythrine pretreatment may be due to effects on phosphate handling. On the other hand, cells preincubated with Rp-cAMP and exposed to bPTH(1-

34) demonstrate a decrease in calcium uptake that is maintained relatively close to basal levels. This inhibition of PKA activity is consistent with the findings of Nemere[36] who observed an increase in PKA activity 5-10 min after bPTH(1-34) treatment of isolated chick enterocytes. Using confocal microscopy, the increase in punctate fluorescence observed with cells preteated with PKA inhibitor at the apical region, extends to the supranuclear region, and indicates that vesicular carriers may be responsible for transporting calcium through the cells.

As observed in the current study, PTH-mediated calcium uptake occurs in isolated intestinal cells cultured in serum; this suggests that circulating serum PTH levels are responsible for this increase in calcium. The response of the cells to a PKA inhibitor supports previous research that the PKA signal transduction pathway is instrumental in PTH-mediated calcium uptake. Visualization of hormone-induced calcium uptake by confocal microscopy illustrates that vesicular transport is involved in this process.

25-Hydroxyvitamin D_3

Although the effects of 25(OH)D_3 on calcium handling for cells in suspension did not support increased uptake in a previous study,[29] evidence was presented for 25(OH)D_3-mediated calcium transport as early as 2 min after steroid treatment in perfused chick duodena.[21] However, cells in suspension did exhibit a 25(OH)D_3–mediated decrease in ^{45}Ca levels,[29] suggesting activation of efflux by the vitamin D metabolite. In the current study, 25(OH)D_3 is shown to increase calcium uptake at 5 and 10 min in these adherent cells, suggesting a role for matrix interactions in directing vectorial movement of ions. When compared to the corresponding time course using confocal imaging, an observed increase in apical fluorescence is apparent. In cells

preincubated with chelerythrine and exposed to $25(OH)D_3$, there are no significant changes in calcium uptake when compared to cells treated with hormone only. Although confocal imaging of cells under these conditions shows no increase in fluorescent intensity, a decrease of fluorescence in the baso-lateral region of the cell and an increase in the apical region are evident. In contrast, cells preincubated with Rp-cAMP demonstrate a decrease in calcium uptake at 10 min and, during confocal imaging, an overall increase in fluorescence is observed along the length of individual cells. These results suggest that the PKA signal transduction pathway is involved with $25(OH)D_3$-mediated calcium uptake and supports the previous work of Phadnis and Nemere[29] in identifying the involvement of the PKA pathway in $25(OH)D_3$–mediated calcium uptake.

Contrary to the results with isolated chick cells in suspension, this study shows that $25(OH)D_3$-mediated calcium uptake does occur in adherent chick enterocytes. Pretreatment of these cells with a PKA inhibitor suggest that the PKA signal transduction pathway is involved maintaining $25(OH)D_3$-mediated calcium uptake after 10 min. The localization of fluorescence at the apical regions of the cells observed in confocal imaging suggests that hormonal changes are facilitated by a vesicular transport mechanism that differs from $1,25(OH)_2D_3$.

Eker et al.[53] have reported that cAMP mediates apical endocytosis in polarized MDCK cells, while Pimplikar and Simons[54] have reported the involvement of PKA. These findings suggest that cAMP or PKA regulates apical endocytosis. The current work supports this research by demonstrating that cAMP or PKA regulates calcium uptake in response to either bPTH(1-34) or $25(OH)D_3$. After noting that cAMP or PKA

is involved with PTH and 25(OH)D_3-mediated calcium uptake, 1,25$(OH)_2D_3$-mediated stimulation of calcium transport also correlates with PKA stimulation.[41]

While strong evidence exists for PKC as the mediator of 1,25$(OH)_2D_3$-stimulated phosphate uptake[35]and transport,[35,41] in chick intestine, phorbol ester has also been found to activate PKA,[35] perhaps through adenylate cyclase[55] and therefore PKC may activate the PKA pathway. Other researchers have reported 1,25$(OH)_2D_3$-mediated activation of MAP kinase.[56] Because of the multiple signaling actions of 1,25$(OH)_2D_3$ and the possibility of "crosstalk," interpretation of inhibitor effects are difficult.

The current work has provided evidence supporting hormone-mediated vesicular transport; but these results do not conclusively implicate vesicular movement of the specific ions, since either kinase activity could conceiveably phosphorylate and activate a transporter existing in the apical membrane, followed by "facilitated diffusion" of the ion. Use of an indicator dye such as fluo-3 in conjunction with confocal microscopy may resolve this. Finally, the confocal data presented here conclusively demonstrate that membrane trafficking is rapid enough to account for mineral transport.

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APPENDICES

Appendix A. Bibliography

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Appendix B. Tables

TABLE 1. PIXEL INTENSITIES* OF EPITHELIAL CELLS TREATED WITH $1,25(OH)_2D_3$

Time	*Apical Region*	*Basal Region*
0 sec	137.45 ± 46.14	169.35 ± 57.56
5	90.77 ± 40.28	100.31 ± 71.36
10	17.95 ± 6.23	53.75 ± 42.39
15	134.89 ± 45.23	165.73 ± 58.46
20	141.14 ± 50.89	162.86 ± 60.79
25	140.31 ± 53.89	172.82 ± 52.80
30	135.17 ± 50.63	178.93 ± 51.22
35	121.81 ± 45.25	172.61 ± 57.42
40	109.11 ± 39.49	144.88 ± 65.20
45	108.28 ± 28.00	139.65 ± 68.95
50	119.68 ± 42.49	155.54 ± 63.02
55	109.89 ± 38.61	143.37 ± 65.24
60	101.91 ± 43.74	77.37 ± 51.12
65	119.49 ± 71.12	103.95 ± 43.58
70	96.28 ± 44.68	76.33 ± 52.22
75	70.24 ± 54.00	98.24 ± 41.98
80	63.84 ± 38.35	91.33 ± 42.42
85	59.49 ± 43.41	85.83 ± 43.49
90	40.39 ± 30.45	78.27 ± 45.28
95	34.17 ± 28.25	79.98 ± 45.94
100	20.36 ± 13.50	63.19 ± 48.52
105	20.92 ± 15.25	63.97 ± 48.72
110	19.14 ± 8.61	48.11 ± 40.65
115	17.98 ± 5.70	44.72 ± 37.70
120	17.19 ± 2.17	45.60 ± 35.24
125	161.92 ± 53.44	192.60 ± 51.93
3 min	163.81 ± 53.23	188.18 ± 55.66
5 min	174.69 ± 52.14	200.77 ± 51.89
7 min	190.59 ± 51.37	207.64 ± 48.90
9 min	203.14 ± 48.27	206.07 ± 52.92

*Values are expressed as mean ± SEM and were analyzed using Adobe Photoshop 6.0.

TABLE 2. PIXEL INTENSITIES* OF EPITHELIAL CELLS TREATED WITH bPTH(1-34)

Time	*Nuclear Region*	*Basal Region*
Control	95.68 ± 21.32	125.51 ± 37.76
Add hormone	99.31 ± 24.13	130.91 ± 41.55
End hormone	98.26 ± 21.63	128.38 ± 38.59
1 min	99.80 ± 22.23	126.77 ± 35.82
3 min	107.92 ± 28.45	132.85 ± 36.29
5 min	108.40 ± 29.00	132.52 ± 36.01
9 min	120.02 ± 28.48	153.24 ± 40.66

*Values are expressed as mean ± SEM and were analyzed using Adobe Photoshop 6.0.

TABLE 3. PIXEL INTENSITIES* OF EPITHELIAL CELLS TREATED WITH $25(OH)D_3$

Time	*Apical Region*	*Basal Region*
Control	105.69 ± 14.10	139.35 ± 22.63
Add hormone	107.10 ± 14.35	137.10 ± 24.95
3 min	103.89 ± 17.82	127.05 ± 24.18
5 min	105.60 ± 17.45	132.63 ± 26.08
7 min	109.58 ± 16.81	138.51 ± 27.57
9 min	109.98 ± 17.04	144.62 ± 30.30

*Values are expressed as mean ± SEM and were analyzed using Adobe Photoshop 6.0.

TABLE 4. PIXEL INTENSITIES* OF EPITHELIAL CELLS PREINCUBATED WITH CHLERYTHRINE AND TREATED WITH $1,25(OH)_2D_3$

Time	*Apical Region*	*Basal Region*
Control	107.01 ± 28.92	131.01 ± 46.80
Add hormone	111.78 ± 30.29	141.69 ± 48.58
10 sec	103.82 ± 30.61	126.13 ± 52.97
1 min	117.24 ± 32.06	137.19 ± 49.51
3 min	122.91 ± 33.52	137.39 ± 51.40
5 min	129.97 ± 36.90	144.89 ± 53.52
7 min	133.39 ± 39.99	150.98 ± 56.22
9 min	141.25 ± 42.88	157.65 ± 57.44

*Values are expressed as mean ± SEM and were analyzed using Adobe Photoshop 6.0.

TABLE 5. PIXEL INTENSITIES* OF EPITHELIAL CELLS PREINCUBATED WITH Rp-cAMP AND TREATED WITH $1,25(OH)_2D_3$

Time	*Apical Region*	*Basal Region*
Control	175.93 ± 47.07	142.41 ± 24.64
Add hormone	184.19 ± 49.90	155.71 ± 31.52
10 sec	184.23 ± 44.40	141.97 ± 35.11
1 min	182.57 ± 52.71	185.51 ± 37.67
3 min	214.98 ± 44.34	237.87 ± 25.63
5 min	231.43 ± 35.75	249.85 ± 14.13
7 min	235.44 ± 34.77	251.50 ± 11.75
9 min	234.87 ± 42.20	251.84 ± 11.99

*Values are expressed as mean ± SEM and were analyzed using Adobe Photoshop 6.0.

TABLE 6. PIXEL INTENSITIES* OF EPITHELIAL CELLS PREINCUBATED WITH Ab099 AND TREATED WITH $1,25(OH)_2D_3$

Time	*Nuclear Region*	*Basal Region*
Control	125.13 ± 25.12	158.86 ± 25.48
Add hormone	132.76 ± 29.43	164.47 ± 26.81
10 sec	133.00 ± 31.24	164.91 ± 25.51
1 min	135.16 ± 27.01	155.40 ± 31.26
3 min	131.23 ± 26.05	146.12 ± 33.33
5 min	126.05 ± 26.08	139.43 ± 33.23
7 min	122.71 ± 25.09	139.68 ± 31.08
9 min	121.11 ± 24.70	136.15 ± 31.03

*Values are expressed as mean ± SEM and were analyzed using Adobe Photoshop 6.0.

TABLE 7. PIXEL INTENSITIES* OF EPITHELIAL CELLS PREINCUBATED WITH CHLERYTHRINE AND TREATED WITH bPTH(1-34)

Time	*Apical Region*
Control	117.97 ± 17.89
Add hormone	120.18 ± 19.85
End hormone	122.07 ± 18.07
1 min	119.92 ± 20.92
3 min	124.87 ± 21.49
5 min	125.50 ± 22.58
9 min	124.18 ± 21.76

*Values are expressed as mean ± SEM and were analyzed using Adobe Photoshop 6.0.

TABLE 8. PIXEL INTENSITIES* OF EPITHELIAL CELLS PREINCUBATED WITH Rp-cAMP AND TREATED WITH bPTH(1-34)

Time	*Apical Region*
Control	154.16 ± 43.71
Add hormone	159.41 ± 46.47
End hormone	175.40 ± 33.84
1 min	183.13 ± 30.38
3 min	198.91 ± 34.32
5 min	212.24 ± 35.86
9 min	228.07 ± 33.58

*Values are expressed as mean ± SEM and were analyzed using Adobe Photoshop 6.0.

TABLE 9. PIXEL INTENSITIES* OF EPITHELIAL CELLS PREINCUBATED WITH CHLERYTHRINE AND TREATED WITH $25(OH)D_3$

Time	*Basal Region*
Control	171.86 ± 40.50
1.4 min	160.89 ± 40.99
3 min	151.40 ± 38.34
5 min	160.26 ± 36.04
7 min	157.12 ± 36.38
9 min	159.66 ± 34.77

*Values are expressed as mean ± SEM and were analyzed using Adobe Photoshop 6.0.

TABLE 10. PIXEL INTENSITIES* OF EPITHELIAL CELLS PREINCUBATED WITH Rp-cAMP AND TREATED WITH $25(OH)D_3$

Time	*Apical Region*	*Basal Region*
Control	124.06 ± 34.29	109.89 ± 31.73
1.4 min	130.28 ± 37.84	118.16 ± 35.36
3 min	140.40 ± 35.67	138.90 ± 39.84
5 min	151.71 ± 40.11	149.53 ± 41.00
7 min	156.30 ± 41.77	154.86 ± 41.50
9 min	160.11 ± 41.59	158.92 ± 41.97

*Values are expressed as mean ± SEM and were analyzed using Adobe Photoshop 6.0.