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Membrane Receptor-Initiated Signaling in $1,25(OH)_2D_3$-Stimulated Calcium Uptake in Intestinal Epithelial Cells

Ramesh C. Khanal, Tremaine M. Sterling Peters, Nathan M. Smith, and Ilka Nemere*

Department of Nutrition and Food Sciences and Center for Integrated BioSystems, Utah State University, Logan, Utah 84322-8700

ABSTRACT

Demonstrating $1,25(OH)_2D_3$-stimulated calcium uptake in isolated chick intestinal epithelial cells has been complicated by simultaneous enhancement of both uptake and efflux. We now report that in intestinal cells of adult birds, or those of young birds cultured for 72 h, $1,25(OH)_2D_3$-stimulates ^{45}Ca uptake to greater than 140% of corresponding controls within 3 min of addition. Such cells have lost hormone-stimulated protein kinase C (PKC) activity, believed to mediate calcium efflux. To further test this hypothesis, freshly isolated cells were preincubated with calphostin C, and calcium uptake monitored in the presence or absence of steroid. Only cells treated with the PKC inhibitor demonstrated a significant increase in ^{45}Ca uptake in response to $1,25(OH)_2D_3$, relative to corresponding controls. In addition, phorbol ester was shown to stimulate efflux, while forskolin stimulated uptake. To further investigate the mechanisms involved in calcium uptake, we assessed the role of TRPV6 and its activation by β-glucuronidase. β-Glucuronidase secretion from isolated intestinal epithelial cells was significantly increased by treatment with $1,25(OH)_2D_3$, PTH, or forskolin, but not by phorbol ester. Treatment of cells with β-glucuronidase, in turn, stimulated ^{45}Ca uptake. Finally, transfection of cells with siRNA to either β-glucuronidase or TRPV6 abolished $1,25(OH)_2D_3$-enhanced calcium uptake relative to controls transfected with scrambled siRNA. Confocal microscopy further indicated rapid redistribution of enzyme and calcium channel after steroid. $1,25(OH)_2D_3$ and PTH increase calcium uptake by stimulating the PKA pathway to release β-glucuronidase, which in turn activates TRPV6. $1,25(OH)_2D_3$-enahnced calcium efflux is mediated by the PKC pathway. J. Cell. Biochem. 105: 1109–1116, 2008.

KEY WORDS: $1,25(OH)_2D_3$; CALCIUM TRANSPORT; INTESTINE; $1,25D_3$-MARRS RECEPTOR/ERp57/PDIA3

The rapid, $1,25(OH)_3D_3$-mediated stimulation of calcium uptake is readily demonstrable in isolated rat intestinal epithelial cells [Nemere and Szego, 1981a,b; Nemere, 2005], but the cell surface receptors involve contributions from both the classical vitamin D receptor (VDR) as well as the $1,25D_3$-MARRS (membrane-associated, rapid response, steroid-binding) receptor. By comparison, the chick intestinal cell model system has been ideal for studying the $1,25D_3$-MARRS receptor-mediated actions, since the classical VDR is not involved in membrane-initiated signaling [Nemere, 2005]. However, investigating rapid, $1,25(OH)_2D_3$-stimulated calcium uptake in normal, vitamin D-sufficient chicks is complicated by simultaneous stimulation of calcium efflux [Nemere and Campbell, 2000]. We postulated that the protein kinase C (PKC) signaling cascade mediates efflux while protein kinase A (PKA) signal transduction mediates uptake on the basis of what was observed in cells from vitamin D-deficient chicks: $1,25(OH)_2D_3$-stimulated efflux of ^{45}Ca in enterocytes from vitamin D-deficient chicks, which lacked hormone-stimulated PKA activity, but retained steroid-stimulated PKC activity [Nemere and Campbell, 2000]. In the current report we present the results of studies in two model systems that demonstrate $1,25(OH)_2D_3$-stimulated calcium uptake in isolated intestinal epithelial cells of vitamin D-sufficient chickens.

MATERIALS AND METHODS

ANIMALS AND CELL ISOLATION

All procedures were approved by the Institutional Animal Care and Use Committee at Utah State University. Adult birds were obtained from the North Logan Poultry Farm; all others were obtained on the day of hatch (Privett Hatchery, Portales, NM) and raised for

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*Correspondence to: Ilka Nemere, Department of Nutrition and Food Sciences, 8700 Old Main Hill, Utah State University Logan, UT 84322-8700. E-mail: ilka.nemere@usu.edu

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3–7 weeks on a vitamin D-supplemented diet (Nutrena Feeds, Murray, UT). On the day of use, animals were anesthetized with chloropent (0.3 ml/100 g body weight) and the duodenal loop removed to ice-cold saline. After chilling for 15 min, the pancreas was removed, the segment everted, and rinsed with additional ice-cold saline. Cells were then isolated in citrate chelation medium at pH 5.0—to maintain viability in culture—as previously described [Nemere et al., 2004]. Cell pellets were collected by centrifugation (500*g*, 5 min, 4°C), and resuspended in 40 ml of Gey's balanced salt solution [Sterling and Nemere, 2005]. The cells were then either used in suspension studies (see below), or cultured in RPMI 1640.

CELL SUSPENSION STUDIES

For calcium uptake studies, 2 μCi $^{45}CaCl_2$ (PerkinElmer Life Sciences, Boston, MA) were added per ml of cell suspension at T = −10 min and basal samples (100 μl) taken at T = −5 and −1 min. Test substances were added at T = 0, and samples taken at 1, 3, 5, 7, and 10 min. Each sample was pipetted into 900 μl of ice-cold Gey's balance salt solution (GBSS), centrifuged (1,000*g*, 5 min, 4°C), the supernatant decanted, and the inside of the tube swabbed with a Kimwipe while still in the inverted position. Cell pellets were lysed in reagent grade water and analyzed for protein by the Bradford assay (BioRad, Hercules, CA) and radioactivity by liquid scintillation spectrophotometry. Test substances included 100 nM phorbol-12-myristate 13-acetate (PMA), 20 μM forskolin, 620 units β-glucuronidase per ml cell suspension, 65 pM bovine PTH(1–34) (all from Sigma Chemical Co., St Louis, MO). For experiments with calphostin C, cells were incubated with 500 nM calphostin C (Alexis Biochemicals, San Diego, CA) for 3 h under fluorescent lights for activation prior to initiation of calcium uptake studies. Experiments with 3 μM RpcAMP (Sigma) used a 30 min incubation period prior to addition of radionuclide. Uptake at each time point was related to cell protein, determined by the Bradford reagent (Bio-Rad Laboratories, Hercules, CA). Subsequently, specific calcium uptake (cpm/mg protein) during the treated phase was normalized to average basal uptake for the corresponding incubation condition.

For β-glucuronidase release studies, 1 ml aliquots of cell suspension were removed at selected times before and after addition of test substances, pipetted into tubes on ice, and cells collected by centrifugation. Supernatant fractions were analyzed using 0.5 mM phenolphthalein-β-D-glucuronide (Sigma) as substrate in 0.125 M Na Acetate, pH 4.5. After a 30 min incubation at 37°C the reaction was stopped by addition of 0.8 M Glycine-NaOH, pH 10.0 and read against phenolphthalein standards.

TISSUE CULTURE STUDIES

Freshly isolated cells were plated in RPMI 1640 (Fisher Scientific, Dallas TX) containing 10% fetal bovine serum (final concentration), and antibiotics (100 units/ml penicillin, 100 mg/ml streptomycin; both from Sigma). After 72 h cells were collected by centrifugation, resuspended in GBSS and used for calcium uptake studies.

For transfection studies, cells were plated in the absence of serum to promote adherence. Forty-four hours later, cells were transfected either with 100 nM siRNA against TRPV6, β-glucuronidase (both from Dharmacon, Lafayette, CO), or scrambled siRNA (Santa Cruz Biotechnology, Santa Cruz, CA) using Simporter (Upstate, Lake Placid, NY). After a 4 h transfection time, media were replaced with RPMI 1640 containing 10% FBS, and culture continued an additional 24 h.

CONFOCAL MICROSCOPY

Freshly isolated intestinal cells from chicks were resuspended in GBSS and cultured overnight with RPMI1640 in dishes containing coverslips. The following morning, media were replaced by GBSS and either vehicle (0.001% ethanol, final concentration), or 300 pM $1,25(OH)_2D_3$ added at T = 0 min. At selected times, media were replaced with 4% paraformaldehyde, 3% sucrose in PBS and the cells fixed for 30 min. After washing with PBS and blocking with normal serum, cells were incubated in the first set of experiments (n = 2) with goat anit-β-glucuronidase (Santa Cruz Biotechnology), followed by fluorescein-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA). Cells were then permeabilized for 5 min with 0.1% Triton X-100 in PBS, washed, and incubated with rhodamine conjugated phalloidin (Sigma Chemical Co.) for 30 min. In the second set of experiments (n = 2), cells were incubated with rabbit anti-TRPV6 (Alamone Labs, Jerusalem, Israel), followed by rhodamine-conjugated secondary antibody (Jackson Immunoresearch), and after permeabilization, incubated with fluorescein-conjugated phalloidin (Sigma).

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 imaging. The krypton-argon laser produced 3 excitation lines of 488, 568, 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 60× oil immersion objective and analyzed for pixel intensity using Adobe Photoshop 6.0 (Adobe Systems, Inc., San Jose, CA).

RESULTS

Our first goal was to verify that $1,25(OH)_2D_3$-stimulated calcium uptake could be observed in chicken intestinal cells lacking hormone-stimulated PKC. We have previously reported that this signal transduction pathway is absent in adult birds [Larsson and Nemere, 2003a,b]. Figure 1A illustrates the results of experiments with cells isolated from adult (58 weeks old) birds. When data were expressed as treated/average basal uptake, control incubations maintained a constant level of ^{45}Ca uptake, whereas treatment of cell suspensions with 130 pM $1,25(OH)_2D_3$ resulted in significantly greater uptake within 3 min after addition. Average maximal uptake in hormone treated cells reached 160–180% of corresponding control levels. Preincubation of cell suspensions with Ab 099 against the N-terminus of the $1,25D_3$-MARRS receptor completely inhibited steroid-stimulated ^{45}Ca uptake (Fig. 1A). An inhibitory effect of Ab 099 on steroid-mediated calcium uptake was also observed in 72 h cultures described below (data not shown). Additional cell preparations from adult birds were tested for the effect of antibody 9A7 against the classical VDR on ^{45}Ca uptake. As shown in Figure 1B, even in the presence of antibody, $1,25(OH)_2D_3$ stimulated radionuclide uptake to 150–165% of corresponding control levels.

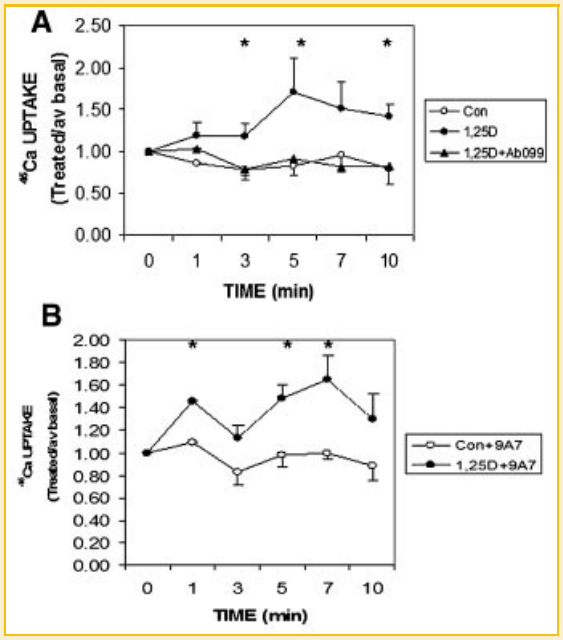

Fig. 1. Calcium uptake in intestinal cells isolated from adult chickens. Cells were isolated from 58-week-old birds by citrate chelation and resuspended in Gey's balance salt solution (GBSS). A portion of the suspension was then added to radionuclide (2 μCi/ml) at T = −10 min, swirled, and further divided into tubes, one of which contained Ab 099 against the 1,25D-MARRS receptor (1/500 dilution, final concentration) (A; n = 4) and two of which contained 2 μg/ml 9A7 against the VDR (B, n = 6). Basal uptake samples (100 μl each) were removed at T = −5 and −1 min, and test substances added at T = 0. Additional samples were taken at the indicated times and pipetted into 900 μl of ice-cold GBSS. Cells were collected by centrifugation, the supernatant decanted, and the inside of the tube swabbed while still in the inverted position. Cells were lysed in water, and aliquots removed for liquid scintillation spectrophotometry and protein analysis. $^{*}P < 0.05$, relative to corresponding control.

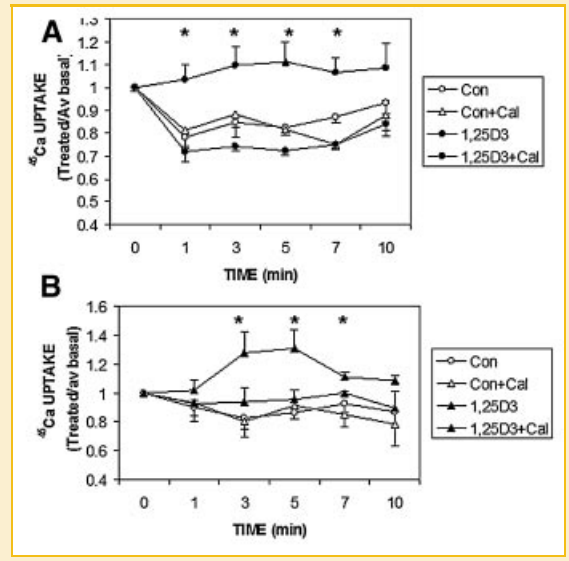

Fig. 2. Effect of calphostin C on calcium uptake in intestinal cells isolated from young birds. Cells were isolated and resuspended in GBSS as described in the legend to Figure 1, and then transferred to 100 mm plastic Petri dishes. One of the two dishes received 500 nM Calphostin C. Cells were then exposed to fluorescent light for 3 h to allow activation of calphostin C. The time course of ^{45}Ca uptake was conducted as described in the legend to Figure 1 with either vehicle plus or minus calphostin C (each, n = 6); 130 pM $1,25(OH)_2D_3$ plus or minus calphostin C (A, each, n = 3); or 650 pM $1,25(OH)_2D_3$ plus or minus calphostin C (B, each, n = 3). $^{*}P < 0.05$, relative to corresponding controls.

Using the PKC inhibitor, calphostin C, allowed demonstration of a similar stimulation of $1,25(OH)_2D_3$ on calcium uptake in intestinal cells isolated from young chicks. As shown in Figure 2, calphostin C had no effect on calcium uptake by control preparations. Treatment of cells with 130 pM $1,25(OH)_2D_3$ resulted in a slight decrease in cellular levels of ^{45}Ca (Fig. 2A), whereas pretreatment of cells with calphostin C, followed by an equivalent concentration of hormone increased radionuclide uptake to 130% of controls. Similar experiments were conducted with 650 pM $1,25(OH)_2D_3$ since this concentration of hormone is optimal for calcium uptake [Nemere et al., 1984], but inhibitory for phosphate uptake [Zhao and Nemere, 2002]. Data presented in Figure 2B indicate that the higher level of steroid caused a slight increase in ^{45}Ca uptake relative to controls, while preincubation with calphostin C followed by hormone resulted in increased calcium uptake levels that were 155% of corresponding controls at T = 3 and 5 min. At 7–10 min after treatment with 650 pM $1,25(OH)_2D_3$, ^{45}Ca levels declined again toward control values, indicating another mechanism for calcium efflux in addition to that mediated by PKC.

In the course of transfection studies we observed that cells isolated from young chicks and cultured for 72 h lost the ability to respond to $1,25(OH)_2D_3$ with stimulated PKC activity (Tunsophon and Nemere, manuscript in preparation). Figure 3A compares the effect of phorbol ester on cells that retain PKC activity (freshly isolated), and 72 h cultures. Direct activation of PKC in freshly isolated cells resulted in a rapid, significant decrease in ^{45}Ca levels to 70% of corresponding controls, while cells cultured for 72 h had calcium levels equivalent to those of corresponding controls (Fig. 3A). In contrast, treatment with 10 μM myo-inositol—the other product of phospholipase C activation—had no effect on calcium uptake or efflux (Fig. 3B).

We subsequently tested 72 h cultured cells for steroid-stimulated calcium uptake. Figure 4A illustrates the results of these experiments. Treatment of cultured intestinal cells with 300 pM $1,25(OH)_2D_3$ increased ^{45}Ca uptake within 5 min to 140% of corresponding control values (Fig. 4A). Preincubation of cells with RpcAMP inhibited steroid-mediated uptake of calcium, while not appreciably altering radionuclide levels in corresponding control incubations (Fig. 4A).

To confirm that PKA mediates calcium uptake, freshly isolated cells were treated with vehicle or 20 μM forskolin and ^{45}Ca uptake monitored. A significant increase in radionuclide levels was seen within 1 min of forskolin addition, reaching 170% of controls after 10 min of incubation (Fig. 4B). Forskolin had a stimulatory effect in 72 h cultures of intestinal cells, while PMA was without effect, as expected (data not shown).

The channel/transporter proteins most likely responsible for calcium uptake in kidney and intestine have been identified [Hoenderop et al., 2003] and designated as TRPV5 and TRPV6,

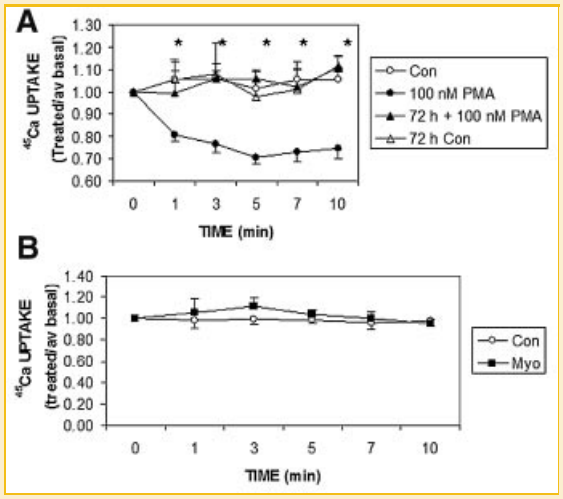

Fig. 3. Effect of phorbol ester or myo-inositol on calcium efflux in intestinal cells isolated from chicks. Procedures were as indicated in the legend to Figure 1. A: At T = 0 min, suspensions were treated with vehicle (0.04% DMSO) or 100 nM PMA (final concentration, n = 14). Alternatively, isolated cells were cultured for 72 h in RPMI 1640–10% FBS prior to experimentation. $^{*}P < 0.05$, relative to corresponding controls. B: At T = 0 min, suspensions of freshly isolated cells were treated with vehicle (water) or 10 μM myo-inositol (final concentration, n = 5).

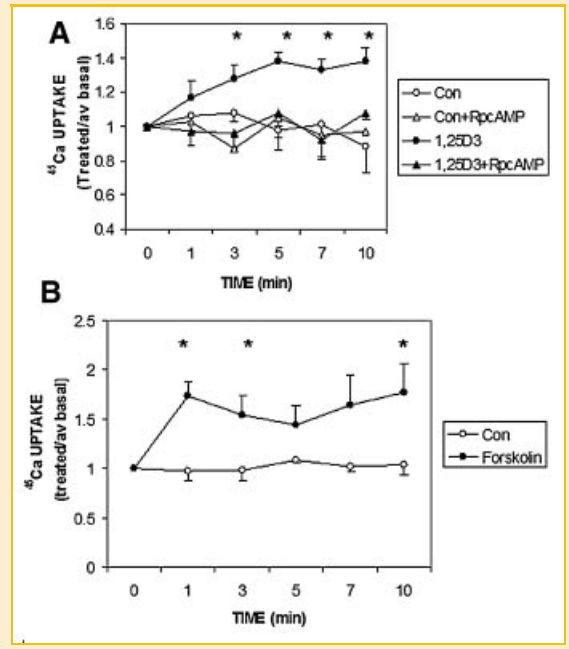

Fig. 4. Stimulation of calcium uptake by $1,25(OH)_2D_3$ in 72 h cultures of intestinal epithelial cells or by forskolin in freshly isolated cells. A: Cells were isolated by citrate chelation and then cultured in 100 mm Petri dishes with RPMI 1640 containing antibiotics and 10% FBS for 72 h. On the day of experimentation, cells were collected by centrifugation, and resuspended in GBSS. Half of the cells were incubated for 30 min with 3 μM RpcAMP and the other half with vehicle prior to initiation of uptake studies; n = 5 $^{*}P < 0.05$, relative to corresponding controls. B: Freshly isolated cells were combined with radionuclide as described in the legend to Figure 1 and treated with either 20 μM forskolin or vehicle (0.04% DMSO); n = 5. $^{*}P < 0.05$, relative to corresponding controls.

respectively. Recently, a classical lysosomal marker enzyme, β-glucuronidase (also known as Klotho), has been reported to activate TRPV5 in kidney [Chang et al., 2005]. We investigated whether compounds known to stimulate calcium uptake in intestinal cells also promoted the release of β-glucuronidase. Figure 5 illustrates the data obtained from these experiments. Both $1,25(OH)_2D_3$ and forskolin produced a significant increase in extracellular β-glucuronidase within 3 min of addition, that reached 130–149% of corresponding controls (Fig. 5A). Parathyroid hormone (bPTH 1–34), which we have previously reported to increase calcium uptake in isolated intestinal epithelial cells [Sterling and Nemere, 2007], also increased β-glucuronidase release, whereas PMA did not (Fig. 5B).

We then incubated isolated intestinal epithelial cells with commercially available β-glucuronidase to determine its effect on calcium uptake. As shown in Figure 6, addition of 620 units (1 mg) of enzyme activity/ml suspension produced a significant increase in ^{45}Ca uptake that reached 130–140% of controls.

As another test of the validity of this pathway in mediating calcium uptake, cells were transfected with scrambled siRNA or siRNA against either TRPV6 or β-glucuronidase, and cultured for a total of 72 h. On the day of use, cells were incubated with radionuclide and either 300 pM $1,25(OH)_2D_3$ or vehicle, and calcium uptake allowed to proceed for 7 min. As indicated in Figure 7, in cultures that were either non-transfected or transfected with scrambled siRNA, hormone treatment resulted in a 145% increase in ^{45}Ca uptake. The steroid did not significantly alter ^{45}Ca uptake in cells transfected with either TRPV6 or β-glucuronidase siRNA (Fig. 7). Western blots confirmed knockdown of the proteins (data not shown).

To verify that these proteins are involved in hormone-stimulated calcium uptake, intestinal cells were cultured on coverslips, treated with vehicle or hormone for selected times, and fixed for confocal microscopy. Non-specific staining controls were negative (data not shown). Figure 8A depicts the distribution of fluorescein, which is indicative of anti-β-glucuronidase localization on the cell surface. Control cells treated for 1, 3, 5, or 10 min with vehicle revealed brush border (arrows) localization of β-glucuronidase as well as faint punctate staining. Brush border localization is identified by rhodamine-conjugated phalloidin (Fig. 8B), while Figure 8C displays merged images of fluorescein and rhodamine. Hormone-treated cells exhibited brush border localization of the enzyme (D1, D3, D5, D10) that was somewhat greater than that observed in controls (Fig. 8A), while punctate staining increased relative to corresponding controls at 5 and 10 min after treatment (Fig. 8A). The merged colors (Fig. 8C) confirm localization of β-glucuronidase in the brush border. These observations were consistent in duplicate experiments.

In Figure 9A the distribution of rhodamine-conjugated secondary antibody to anti-TRPV6 is shown, while Figure 9B illustrates fluorescein-conjugated phalloidin, and Figure 9C displays the merged images. Control cells (C1, C5, C7, C10) display light punctate labeling representative of TRPV6, while $1,25(OH)_2D_3$ treated cells

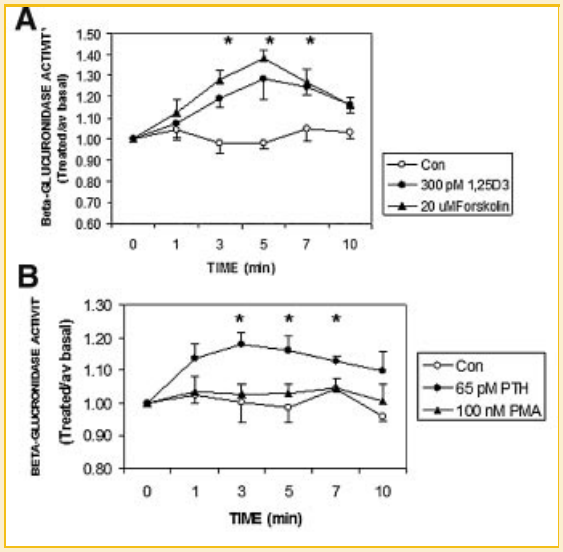

Fig. 5. Effect of hormones and protein kinase activators on β-glucuronidase release. Cell isolation was as described in the legend to Figure 1. Cells were resuspended in GBSS and partitioned into separate incubation vessels. After removal of baseline aliquots (1 ml each), test substances were added, and additional aliquots removed at the indicated times and chilled on ice. After centrifugation, supernatants were analyzed for β-glucuronidase activity and pellets for cell protein to yield enzyme specific activity. Specific activity in the treated phase was normalized to baseline values. A,B: n = 6 for each treatment group; $^{*}P < 0.05$, relative to corresponding controls.

rapidly (1 min) exhibited increased intensity of punctate staining (D1), and at 7 min of incubation, noticeably more TRPV6 labeling is evident in brush borders (Fig. 9B), coinciding with optimum levels of steroid-stimulated calcium uptake. At 10 min after steroid hormone, TRPV6 labeling of the brush border has decreased, paralleling the decline in $1,25(OH)_2D_3$-mediated calcium uptake. Punctate staining along the length of the cell (D10, Fig. 9C) remains visible.

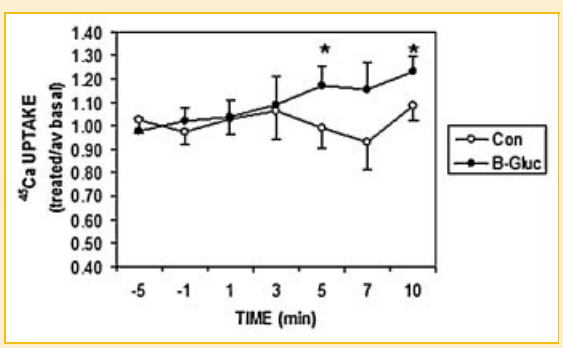

Fig. 6. Exogenous β-glucuronidase stimulates calcium uptake in isolated intestinal epithelial cells. Procedures were as described in the legend to Figure 1. At T = 0, cell suspensions were either treated with vehicle (water) or 1,240 units (1 mg/ml) of β-glucuronidase. n = 8, $^{*}P < 0.05$, relative to corresponding controls.

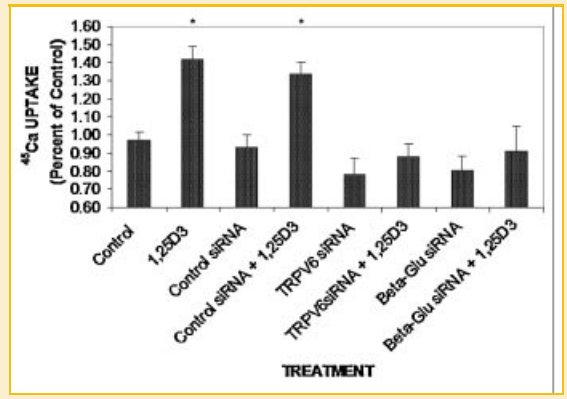

Fig. 7. siRNA against TRPV6 or β-glucuronidase inhibits $1,25(OH)_2D_3$-stimulated calcium uptake. Cells were isolated and plated in 35 mm dishes without serum to promote adhesion. The next day cells were transfected for 5 h, and then media containing 10% FBS added and the cultures continued for a total of 72 h prior to experimentation. On the day of use, media were aspirated and replaced with GBSS-BSA containing $^{45}CaCl_2$ and either vehicle or 300 pM $1,25(OH)_2D_3$. After 7 min incubation, media were removed and the plates washed three times with 4 ml of cold GBSS. Cells were then collected, lysed and analyzed for radioactivity and protein. n = 7; $^{*}P < 0.05$, relative to controls.

DISCUSSION

Our findings indicate there are two viable model systems for studying calcium uptake in isolated intestinal epithelial cells of chickens: cells isolated from adult birds and cells isolated from chicks that have been cultured for 72 h. Both are made possible by the loss of hormone-stimulated PKC activity (that mediates the rapid efflux of calcium), but retention of steroid-stimulated PKA activity responsible for calcium uptake. In spite of the central role of PKC in $1,25(OH)_2D_3$-mediated calcium efflux, evidence for an alternative mechanism of cation extrusion was evident. In cells treated with 650 pM $1,25(OH)_2D_3$ in the presence of calphostin C ^{45}Ca levels declined toward basal values within 10 min. A role for PKC has been well documented in vesicular exocytosis [Cosin-Binker et al., 2007]. Thus, the data support the vesicular transport model for calcium absorption [Warner and Coleman, 1975; Bikle et al., 1981; Davis and Jones, 1981, 1982; Nemere et al., 1986; Nemere and Norman, 1988], but they are also indicative of a back up mechanism to prevent accumulation of toxic levels of the divalent cation. A number of workers believe in a model where calcium is "ferried" through the cytoplasm to be extruded by a basal-lateral membrane Ca^{2+}-ATPase, which may be operative as the back up mechanism. However, the main function of the Ca^{2+}-ATPase may be to decrease signaling calcium that enters through voltage-gated channels in the basal lateral membrane [de Boland et al., 1990], and which in turn are involved in rapid responses to $1,25(OH)_2D_3$.

We have also demonstrated that the $1,25D_3$-MARRS receptor, and not the VDR, is responsible for initiating signal transduction in both calcium and phosphate uptake in chicken intestinal cells [Nemere et al., 2004; Khanal et al., 2007]. We have noted elsewhere that in rat intestinal cells, both receptors appear to be involved in membrane-initiated signaling [Nemere, 2005].

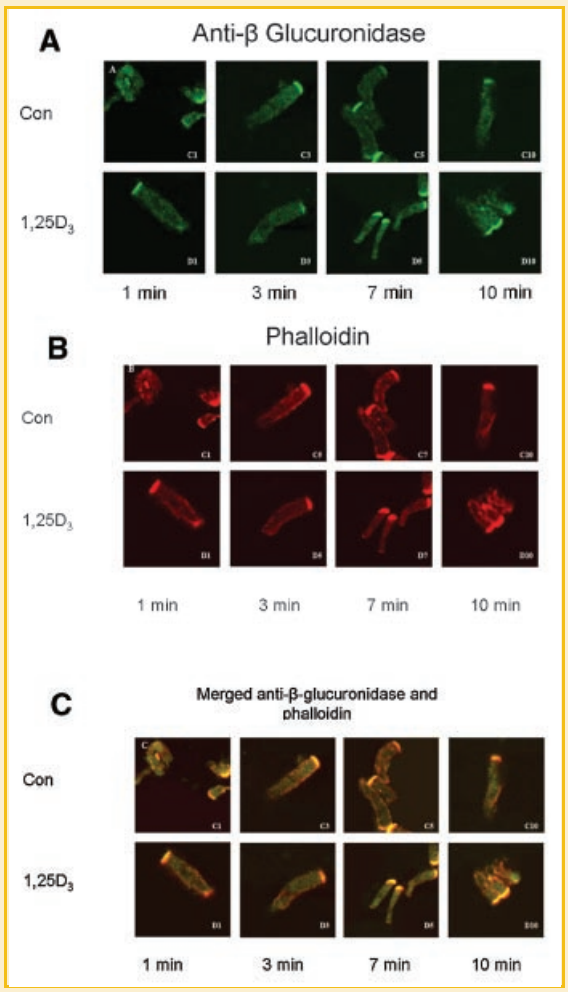

Fig. 8. Immunocytochemical localization of β-glucuronidase as a function of time after $1,25(OH)_2D_3$. Intestinal cells were isolated from chicks by citrate chelation, resuspended in GBSS, and cultured with RPMI1640 in tissue culture dishes containing a coverslip. The following morning, media were aspirated, and replaced with GBSS. At T = 0 min, half of the dishes were treated with vehicle (0.001% ethanol, final concentration), or 300 pM $1,25(OH)_2D_3$. At the indicated times, media were aspirated and replaced with 4% paraformaldehyde, 3% sucrose in PBS and fixed for 30 min. After washing and blocking with normal serum, cells were overlayed with primary antibody for 60 min, washed, and exposed to secondary FITC-conjugated antibody for 30 min. After additional washes, the cells were permeabilized for 5 min with 0.1% Triton X-100 in PBS, washed again, and incubated with rhodamine-conjugated phalloidin (30 min). After three final washes, coverslips were mounted. A: Fluorescein labeled cell surface β-glucuronidase in controls at 1, 3, 5, 10 min (C1, C3, C5, C10, respectively) and $1,25(OH)_2D_3$-treated cells (D1, C3, D5, D10). B: Rhodamine conjugated phalloidin (actin). C: merged images. Arrows indicate a brush border for orientation.

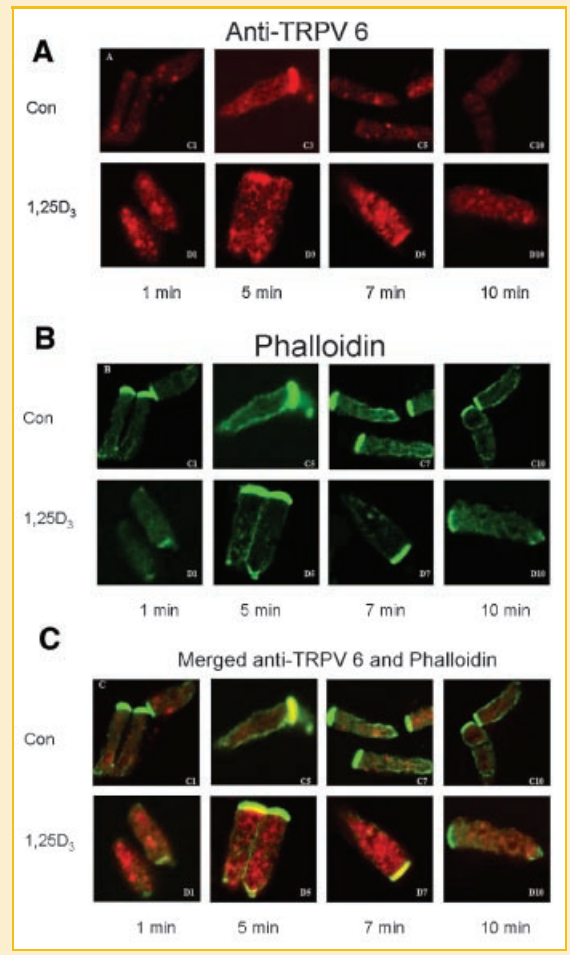

Fig. 9. Immunocytochemical localization of TRPV6 as a function of time after $1,25(OH)_2D_3$. Cells were prepared as described in the legend to Figure 8, and stained with rhodamine-conjugated anti-TRPV6, followed by fluorescein-conjugated phalloidin after permeabilization. A: Rhodamine labeled cell surface TRPV6 in controls (C1, C5, C7, C10) and hormone treated cells (D1, D5, D7, D10); (B) fluorescein-labeled phalloidin in the same cells; (C) merged images. Arrows indicate a brush border for orientation.

One of the rapid responses initiated by both $1,25(OH)_2D_3$ and PTH is the exocytosis of a number of lysosomal enzymes including cathepsin B [Nemere and Szego, 1981 a,b; Nemere and Norman, 1991] acid phosphatase, and *N*-acetyl-β-D-glucosaminidase [Nemere and Szego, 1981a,b]. In the current work we further demonstrate that both the steroid and peptide hormones increase the release of β-glucuronidase. It has long been postulated that the release of such enzymes could be responsible for modification of the cell surface [Szego and Pietras, 1984], although the specific activation of the TRPV channel has been identified only recently [Chang et al., 2005]. We have previously demonstrated that PKA signaling is involved in PTH-enhanced calcium uptake [Sterling and Nemere, 2007]. It is now clear that the initial pathway, after binding of either PTH or $1,25(OH)_2D_3$ to their respective cell surface receptors, involves activation of adenylate cyclase and subsequently PKA, which then mediates the release of β-glucuronidase that activates TRPV6. This sequence of events may account for the lack of $1,25(OH)_2D_3$-mediated rapid calcium transport in vitamin D-deficient animals: up regulation of adenylate cyclase expression must occur before enhanced calcium transport can occur [Corradino, 1974; Nemere and Campbell, 2000]. In cells from vitamin D-replete chicks, the rapid cell surface appearance of TRPV6 immunoreactivity suggests that the transporter is held in vesicles below the plasma membrane for rapid insertion as signaled by hormone.

Although calcium transport is often described as occurring by facilitated diffusion, this model has yet to be reconciled with the observations that (1) the time course of increasing and then decreasing lysosomal content of ^{45}Ca after a single dose of $1,25(OH)_2D_3$ in rachitic chicks exactly parallels net calcium absorption [Nemere and Norman, 1988]; and (2) the lysomotropic agent chloroquine completely blocks $1,25(OH)_2D_3$-stimulated transport of the cation [Nemere et al., 1986]. With regard to the vesicular model of transport, it remains to be established how activation of the TRPV transporter/channel promotes vesicle filling. One possibility is exocytotic insertion of a calcium channel that constrains compensatory endocytosis to the sites of exocytosis, as demonstrated in sea urchin eggs [Smith et al., 2000]. In intestine this would translate into direct filling of transport vesicles that are known to contain calbindin D_{28k} [Nemere et al., 1991], and which subsequently fuse with lysosomes [Nemere and Norman, 1988; Nemere et al., 1991]. Another possibility is distribution of calcium from the brush border channel/transporter directly to the endoplasmic reticulum [Larsson and Nemere, 2002; Lalevee et al., 2003; Lencesova et al., 2004], which could then bud off transport vesicles. Indeed, an early role for the endoplasmic reticulum was noted in time course studies of vesicular transport [Nemere and Norman, 1988]. Clearly, any model of transepithelial calcium movement must eventually account for all observations made over the years. Recently, Benn et al. [2008] have reported that mice having both calbindin D_{9k} and TRPV6 knockouts have normal calcium homeostasis. This is not at odds with the present work, in that there may be species differences in the preferred calcium transporter, and/or TRPV6 may be the predominant transporter responding to $1,25(OH)_2D_3$, as demonstrated by short term siRNA studies, but back up mechanisms may exist in long term knockout conditions.

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