1,25-dihydroxyvitamin D₃ stimulates vesicular transport within 5 s in polarized intestinal epithelial cells

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Abstract

Controversy remains regarding whether the seco-steroid hormone 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) enhances calcium and phosphate movement across the intestinal epithelial cell by facilitated diffusion or a vesicular transport mechanism. In this study we investigated whether membrane trafficking, as judged by confocal microscopy, was sufficiently rapid in comparison to hormone-stimulated uptake of phosphate (³²P). Primary cultures of chick intestinal cells were established overnight either in Petri dishes (uptake studies) or chambered coverslips (confocal microscopy). Addition of 130 pM 1,25(OH)₂D₃ resulted in an apparent increase in ³²P uptake within 1 min, relative to controls, that was statistically significant from 3–10 min of incubation. Using the endocytic marker dye, FM1-43, confocal microscopy revealed a profound decrease in membrane-associated fluorescence (apical>basal) within 10 s of hormone treatment, a return of fluorescence at 15–65 s, followed by

another round of decreasing and increasing fluorescence. Between 3–9 min of incubation, fluorescence intensity increased 50% (apical region) and 20% (basal region) over control conditions. An antibody (Ab 099) directed against a putative membrane receptor for 1,25(OH)₂D₃ (1,25D₃-MARRS) inhibited both ³²P uptake, and changes in fluorescence. In addition, the protein kinase C (PKC) inhibitor, calphostin C, inhibited both ³²P uptake and the observed 1,25(OH)₂D₃-mediated changes in fluorescence. At the microscopic level, calphostin C pretreatment abolished the very rapid redistribution of the endocytic marker dye, although a slight increase in fluorescence was still observed. We conclude that 1,25(OH)₂D₃-stimulated vesicular trafficking is mediated by the 1,25D₃-MARRS protein, implicates a PKC signaling mechanism, and occurs in a time frame that is commensurate with a role in ion transport.

Journal of Endocrinology (2005) **185**, 81–91

Introduction

In intestinal cells from young (3–7 weeks), vitamin D replete chicks, the primary effect of the steroid hormone 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) appears to be on phosphate uptake (Zhao & Nemere 2002) and transport (Nemere 1996a), mediated in part by protein kinase C (PKC; Zhao & Nemere 2002, Larsson & Nemere 2003) rather than calcium uptake (Nemere & Campbell 2000) and transport (Nemere 1996a).

Phosphate uptake at the apical region of the enterocytes is mediated by a Na/P_i cotransporter (Murer *et al.* 2001). However, phosphate accumulation has been reported to occur in lysosomes of fibroblasts (Pisoni 1991), as well as intestinal epithelial cells exposed to transport conditions (Nemere 1996b), suggesting that vectorial movement of the anion occurs in vesicles. Moreover, the transporter itself has been found to be internalized under low uptake conditions (Murer *et al.* 2001) and is presumably reinserted into the plasma membrane when phosphate transport is increased.

In the current study, we use primary cultures of chick intestinal epithelial cells to assess the time course of ³²P uptake as stimulated by 1,25(OH)₂D₃, and compare that to the time course of vesicular trafficking with confocal microscopy. Our results indicate that 1,25(OH)₂D₃-mediated vesicular trafficking is sufficiently rapid to play a role in phosphate uptake or transport.

Materials and Methods

Surgical procedures and cell isolation

All surgical procedures were approved by the Institutional Animal Use and Care Committee at Utah State University (Logan, UT, USA). White leghorn cockerels (Privett Hatchery, Portales, NM, USA) 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, USA). On the day of use, chicks were anesthetized with chloropent (0.3 ml/100 g 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 in chilled saline solution.

The chick enterocytes were isolated with citrate chelation media (Nemere & Campbell 2000, Zhao & Nemere 2002) adjusted to pH 5.0 to promote viability and retention of morphology (Caldwell *et al.* 1993). The cells were collected by low speed centrifugation (500 g, 5 min, 4 °C) and resuspended in a small volume of Gey's balanced salt solution (GBSS; containing 119 mM NaCl, 4.96 mM KCl, 0.22 mM KH₂PO₄, 0.84 mM NaHPO₄, 1.03 mM MgCl₂·6H₂O, 0.28 mM MgSO₄·7H₂O, 0.9 mM CaCl₂, pH 7.3). Aliquots of the cell suspension (1 ml) were pipetted into 35 mm plastic Petri dishes (Falcon, Fisher Scientific, Houston, TX, USA) containing 3 ml of RPMI-1640 medium (Hyclone, Logan, UT, USA) and antibiotics (100 units/ml penicillin, 100 mg/ml streptomycin (both from Sigma)). The cells were incubated for 24 h (in the absence of serum) at 37 °C with 5% CO₂/95% air to promote cell adhesion.

Time course of phosphate uptake

After cells were cultured as described above, the media were aspirated and replaced with GBSS containing radionuclide (2 µCi/ml H₃³²PO₄; New England Nuclear, Boston, MA, USA) at 23 °C. After each dish was exposed to GBSS with radionuclide for 10 min, dishes for the zero time point were washed three times each 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 × -100). The remaining dishes were treated with 0.01% ethanol (final concentration) for vehicle controls or 130 pM 1,25(OH)₂D₃ for hormone treated cells. At designated time intervals after hormone addition, the media were aspirated and the cells washed and then lysed in preparation for protein determination by the Bradford Assay (BioRad, Hercules, CA, USA) against bovine γ-globulin as standard (Sigma) and radionuclide measurement by liquid scintillation spectrophotometry. The c.p.m. 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.

Statistical comparisons between controls and treated samples were performed using the Student's *t*-test for unpaired observations or SAS 9.1 for ANOVA; significant differences were determined with a 95% probability.

Effect of inhibitors on phosphate uptake

The role of the 1,25D₃-MARRS protein (membrane associated, rapid response steroid-binding protein – a putative membrane receptor for 1,25(OH)₂D₃) was analyzed using a highly specific polyclonal antibody (Ab 099; Nemere *et al.*, 2000, 2004) generated by the multiple

antigenic peptide format to the N-terminal sequence of the protein (Center for Integrated BioSystems, Logan, UT, USA). After cells were cultured as described above, the media in each dish were aspirated and replaced with GBSS/0.1% BSA with or without 1/500 dilution of Ab 099 and preincubated for 5 min prior to experimentation. After cell cultures were overlaid with GBSS containing radionuclide (2 µCi/ml H₃³²PO₄) for 10 min, each dish was exposed to vehicle or hormone for 7 min. The media were aspirated and the cells washed and then lysed in preparation for analysis as described above.

To assess the role of PKC, adherent cells were preincubated with or without 500 nM calphostin C (Alexis Biochemicals, San Diego, CA, USA) for 3–3.5 h (Kobayashi *et al.* 1989, Bruns *et al.* 1991) and then incubated with radionuclide with or without hormone as described for the time course of phosphate uptake.

Confocal microscopy

Vesicular movement was examined using confocal microscopy with the probe FM 1–43, which is nonfluorescent in the aqueous environment of the medium but fluoresces once it inserts into the plasma membrane. A MRC 1024 laser-scanning confocal microscope system mounted in the Keller position and attached to a Nikon TE-200 microscope (BioRad) 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 (BioRad), using a 60x oil immersion objective and analyzed for pixel intensity using Adobe Photoshop 6.0 (Adobe Systems, Inc, San Jose, CA, USA).

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 antibiotics and cultured overnight at 37 °C with 5% CO₂/95% air. FM 1–43 (Molecular Probes, Eugene, OR, USA) was used to label membranes; this probe was excited by the 488 nm laser line with maximum emission spectra at 625 nm. LysoTracker-red (Molecular Probes) was used for lysosomal visualization; this label was excited at 568 nm laser line with 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 overlaid with GBSS-0.1% BSA only for vehicle or GBSS-0.1% BSA with hormone for treated cells. For inhibitor studies, cells were preincubated with 1/500 dilution Ab 099 (5 min at 23 °C) or 500 nM calphostin C (3–3.5 h at 23 °C under fluorescent light) prior to addition of fluorescent label.

The images were then captured at 5-s intervals for 10 min using a 60x oil immersion objective and analyzed for pixel intensity as described above.

Figure 1 Time course of 1,25(OH)₂D₃-mediated phosphate (³²P) uptake in isolated intestinal epithelial cells. Enterocytes were isolated by a chelation protocol, collected by low speed centrifugation, and resuspended in GBSS. One milliliter aliquots of cell suspensions were seeded into 35 mm Petri dishes containing 3 ml RPMI-1640 medium and incubated overnight (37 °C with 5% CO₂/95% air) to allow cells to adhere. For uptake studies, media were aspirated and replaced with 2 μCi ³²H₃PO₄/1 ml of GBSS. Cells were treated with 130 pM 1,25(OH)₂D₃ (●) 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 and radioactivity analysis by liquid scintillation spectrophotometry. Data were calculated as c.p.m./μg protein and then related to corresponding basal levels. Values represent means ± S.E.M. (n=6). Significant differences (*P<0.05), determined using the student *t*-test, are relative to corresponding controls.

Results

Effect of 1,25(OH)₂D₃ on phosphate uptake

Figure 1 illustrates the results of time course studies on ³²P uptake. In controls, a time dependent decrease in ³²P levels was observed. Hormone-treated cells also exhibited a time dependent decline in ³²P levels, but were consistently higher than the corresponding controls. We surmise that this increase represents steroid-mediated uptake. Thus, 130 pM 1,25(OH)₂D₃ stimulated an apparent increase in ³²P uptake within 1 min when compared with controls. In the controls, the corresponding ³²P values (c.p.m./μg protein compared to basal levels, means ± S.E.M.) 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)₂D₃, ³²P uptake increased by approximately 203%, 216%, 204%, 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)₂D₃ on distribution of FM 1–43

An equivalent time course was used in studies with confocal microscopy of cells labeled with FM 1–43. This dye, which is non-fluorescent in an aqueous environment, fluoresces when associated with a membranous 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 s of image capture after which the steroid was added.

Within 5 s of 1,25(OH)₂D₃ addition, a decrease in apical fluorescence was observed (Fig. 2B) with an even more dramatic decrease at 10 s (Fig. 2C). The intensity of punctate fluorescence increased markedly after 15 s (Fig. 2D). These very rapid effects were observed in three independent experiments. In subsequent frames (Figs 2F–M), apical fluorescence appeared punctate while basal staining appeared continuous. Between 1 min and 1 min 25 s after 1,25(OH)₂D₃ addition, the staining pattern became more punctate at supranuclear localizations (Figs 2M–R). A loss of overall fluorescence was again observed between 1 min 30 s and 2 min 5 s after hormone addition, followed by a dramatic increase in fluorescence 5 s 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)₂D₃ appeared to flicker. For the remainder of the time course (3–9 min after 1,25(OH)₂D₃ addition), an overall increase in fluorescence was observed (Figs 2BB–EE). The observed changes in fluorescence at the apical (Fig. 3; diamond symbols) and basal regions (Fig. 3; circles) are quantitatively verified by pixel intensity for each confocal image.

A second dye, Lyso-Tracker Red, was also tested in time course studies using confocal microscopy. This dye, which is non-fluorescent at a neutral pH, fluoresces when incorporated into acidic organelles. Studies with Lyso-Tracker Red suggested increased movement of labeled organelles after hormone addition (data not shown), but definitive patterns of movement were not evident.

Effect of Ab 099 on 1,25(OH)₂D₃ -mediated ³²P uptake

To assess the role of a putative membrane receptor, 1,25D₃ MARRS protein, on phosphate uptake, cultured cells were preincubated in the absence or presence of Ab 099. Radionuclide and either vehicle or hormone was then added and incubation continued an additional 7 min. Figure 4 illustrates that 130 pM 1,25(OH)₂D₃ increased ³²P uptake to 170% of controls (1,25D₃; solid bar). Pre-incubation of cells with Ab 099 (Ab only; open bar) showed no significant difference in ³²P uptake when compared with controls without Ab 099 but completely inhibited hormone stimulated uptake (Ab1,25D₃; striped bar) when compared with cells treated with 1,25(OH)₂D₃ alone.

Figure 2 (A)–(O).

Figure 2 (P)–(AA).

Figure 2 (BB)–(EE).

Figure 2 Effect of 1,25(OH)₂D₃ on endocytic distribution of FM 1–43 ($n=2$). Confocal images are presented of cells labeled with FM 1–43, then treated with 1,25(OH)₂D₃. Cells were plated overnight in chambered coverslips. The dye was diluted into GBSS, which was then overlayed onto the cells. Since no changes in fluorescence distribution occurred in controls over a 10 min period (not shown), subsequent experiments used a 1 min control period for image capture, after which GBSS containing hormone was added to the medium and additional images were captured at 5 s intervals for 10 min using a 60x oil immersion objective. Outlined areas were analyzed for pixel intensity using Adobe Photoshop 6.0. The emission and excitation wavelengths were 488 and 522 nm, respectively. (A) control; (B–AA) every 5 s after hormone addition; (BB) 3 min; (CC) 5 min; (DD) 7 min; (EE) 9 min. The arrow in this and subsequent micrographs indicates the apical surface.

Effect of Ab 099 and 1,25(OH)₂D₃ on distribution of FM 1–43.

Preincubation of cells with Ab 099 also abolished rapid (5–60 s) 1,25(OH)₂D₃-mediated changes in FM 1–43 distribution (data not shown), but as indicated in Fig. 5 some relocalization of dye occurred as judged by the disappearance of nuclear outlines in the two cells depicted between 1–9 min after hormone addition (Figs 5C–G). The pixel intensities given in Fig. 6 indicate that changes observed in the nuclear (triangles) and basal (circles) regions of the confocal images were subtle.

Effect of PKC inhibitor calphostin C on 1,25(OH)₂D₃-mediated ³²P uptake

To identify the involvement of PKC in 1,25(OH)₂D₃-mediated phosphate uptake, cultured cells were preincu-

bated with or without calphostin C before exposure to hormone (Fig. 7). Figure 7 illustrates the results of time course studies on ³²P uptake in the presence of inhibitor. Whereas the steroid hormone (closed circles) stimulated phosphate uptake relative to controls (open circles), pretreatment of cells with calphostin C abolished such 1,25(OH)₂D₃-mediated enhancement (closed triangles). Statistical comparison by ANOVA indicated that 1,25(OH)₂D₃-treated cells exhibited significantly greater uptake relative to both controls and calphostin-pretreated cells ($n=5$, $P<0.05$ for $T=1-5$ and 10 min).

Effect of calphostin C on distribution of FM 1–43

Pretreatment of cells with calphostin C effectively blocked the very rapid (5–60 s) 1,25(OH)₂D₃-mediated changes in FM 1–43 fluorescence (data not shown). Instead, a gradual

Figure 3 Pixel intensities for confocal images of representative epithelial cells treated with 1,25(OH)₂D₃. Outlined areas in Fig. 2 were analyzed for pixel intensity using Adobe Photoshop 6.0. Values for apical (●) and basal (◆) intensities are expressed as means $\pm$ S.D. ($n=4$ for each).

increase in cellular fluorescence became noticeable at the apical and basal regions of the cells after 10 min (compare Figs. 8A–8 G). Although these slight changes in fluor-

escence were observed, quantitative analysis of the pixel intensities indicated that these effects were not significantly different (Fig. 9).

Figure 4 Comparisons of the effects of 1,25(OH)₂D₃ and Ab 099, alone or in combination, on phosphate (³²P) uptake in isolated chick enterocytes. Cells were pretreated with 1/500 dilution of Ab 099 (Ab) for 5 min at 23 °C prior to addition of radionuclide and either 130 pM 1,25(OH)₂D₃ (1,25D) or vehicle (0.01% ethanol, final concentration) for a total of 7 min. Experimental procedures for ³²P uptake experiments are described in Fig. 1. Data were calculated as c.p.m. and related to corresponding basal values. All values were normalized to % control for final comparisons and are represented at means $\pm$ S.E.M. ($n=9$).

Discussion

Previous experiments with isolated chick enterocytes in suspension have demonstrated that 1,25(OH)₂D₃ increases phosphate uptake as early as 5 min (Zhao & Nemere 2002). In the present study with cultured chick enterocytes, an increase in 1,25(OH)₂D₃-mediated phosphate uptake occurs as early as 1 min and is significantly higher than controls at 3 min. Although the treated/average basal values decreased with time, in both hormone-treated cells when compared with basal, these levels remain closer to basal values than those reported for cells in suspension. Perhaps as a consequence, the increase in ³²P uptake is sustained at an average of 208% of controls from 3 to 10 min compared with 151% of controls observed at 10 min with cells in suspension (Zhao & Nemere 2002).

Confocal imaging of these cells indicates that membrane trafficking occurs as early as 5 s after hormone addition. The extremely rapid decrease in apical fluorescence that is observed within 10 s of 1,25(OH)₂D₃ treatment has, however, two interpretations. The results could suggest an endocytotic mechanism that is consistent with the findings of Warner & Coleman (1975). This study followed 1,25(OH)₂D₃-mediated calcium uptake in vitamin D-deficient chicks using electron probe analysis, and suggested calcium may be endocytosed just below the

Figure 5 Effect of Ab 099 and $1,25(\text{OH})_2\text{D}_3$ on endocytic distribution of FM 1–43 labeling ($n=2$). Confocal images were obtained of cells preincubated with Ab 099 at 1/500 dilution, labeled with FM1–43, then treated with 130 pM $1,25(\text{OH})_2\text{D}_3$. Cells were plated overnight in chambered coverslips. The dye was diluted into GBSS, which was then overlaid onto the cells. After a 1-min incubation with the dye alone GBSS containing hormone was added and images were captured and analyzed as described in Fig. 2. (A) control; (B) 10 s; (C) 1 min; (D) 3 min; (E) 5 min; (F) 7 min; (G) 9 min.

brush border, appearing as discreet localizations in the apical region, followed by extrusion at the lateral membrane. Our observations are also consistent with delivery of unlabeled, Golgi-derived vesicles, perhaps containing the

Na/P_i transporter. In kidney cells, Murer *et al.* (2001) have reported internalization of the transporter when parathyroid hormone (PTH) is withdrawn. Such sequestration mechanisms are known to be rapidly reversible and would

Figure 6 Pixel intensities for confocal images of representative epithelial cells preincubated with Ab 099 and treated with 1,25(OH)₂D₃. Outlined areas in Fig. 5. were analyzed for pixel intensity using Adobe Photoshop 6.0. Values for the basal (●) and nuclear (▲) regions are expressed as means $\pm$ s.d. ($n=2$, for each).

be amenable to testing with an antibody against the transporter. The sustained increase in fluorescence intensity between 1–9 min after 1,25(OH)₂D₃ addition suggests repeated endocytotic/exocytotic events, and can be attributed to labeling of existing membrane, new membrane, and probe trapped within vesicles (Ryan *et al.* 1996). To determine the signal transduction events that may facilitate the vectorial transport of phosphate ions, Ab 099 (against the 1,25D₃ MARRS protein) and a PKC inhibitor (calphostin C), were employed in uptake experiments for the current study.

Using Ab 099, Nemere *et al.* (2000) have immunohistochemically characterized a putative plasmalemmal recep-

Figure 7 Inhibition of 1,25(OH)₂D₃-mediated ³²P uptake by calphostin C in isolated intestinal epithelial cells. Enterocytes were isolated by a chelation protocol, collected by low speed centrifugation, and resuspended in GBSS. Cells were pretreated with vehicle (○), 500 nM calphostin C followed by hormone (▲), or with 130 pM 1,25(OH)₂D₃ only (●). Experimental procedures for ³²P uptake experiments were described in Fig. 1. Values represent means $\pm$ S.E.M. ($n=7$). Significant differences (* $P<0.05$) were determined using SAS 9.1 for ANOVA and are relative to cells treated with 130 pM 1,25(OH)₂D₃ only (●).

tor, 1,25D₃ MARRS protein. In addition to these findings, several studies have demonstrated that 1,25D₃ MARRS protein is responsible for binding 1,25(OH)₂D₃ in the baso-lateral membrane (Nemere *et al.* 2000, 2004). In the current work, preincubation of isolated intestinal epithelial cells with Ab 099 completely inhibited 1,25(OH)₂D₃-mediated phosphate uptake. Using equivalent conditions for confocal microscopy, no hormone-mediated increase in fluorescence or redistribution of perinuclear fluorescence occurred. In essence, pretreatment of cells with Ab 099 inhibits 1,25(OH)₂D₃-induced changes.

Earlier studies have indicated that PKC is most likely involved in phosphate uptake: phorbol ester, but not forskolin, stimulates ³²P uptake in enterocytes (Zhao & Nemere 2002); and the dose-response curves for 1,25(OH)₂D₃-stimulated phosphate transport parallels PKC activation (Larsson & Nemere 2003). In the current study, pretreatment of cultured cells with a PKC inhibitor (calphostin C) abolished steroid-enhanced uptake of phosphate. Although slight increases in fluorescence are observed in these confocal images, the presence of calphostin C significantly inhibited the membrane trafficking that is apparently involved with 1,25(OH)₂D₃-mediated phosphate uptake.

In conclusion, 1,25(OH)₂D₃ stimulated exceedingly rapid membrane trafficking that is directly involved in phosphate uptake and transport. The 1,25D₃-MARRS protein mediates hormone-induced signal transduction, including activation of PKC (Nemere *et al.* 2000, 2004). Since phosphate transport is critical in young growing animals to support rapid bone development, the 1,25D₃-MARRS protein (Nemere *et al.* 2004) may represent a new therapeutic target for amelioration of bone loss.

Figure 8 Effect of calphostin C and $1,25(\text{OH})_2\text{D}_3$ on endocytic distribution of FM 1-43 labeling ($n=2$). Confocal images are presented of cells preincubated for 3 h with a PKC inhibitor (500 nM calphostin C), labeled with FM 1-43, then treated with 130 pM $1,25(\text{OH})_2\text{D}_3$. Cells were plated overnight in chambered coverslips. Procedures were as indicated in the legend to Fig. 2 for addition of the dye and hormone, and image capture. (A) control; (B) 10 s; (C) 1 min; (D) 3 min; (E) 5 min; (F) 7 min; (G) 9 min.

Figure 9 Pixel intensities for confocal images of representative epithelial cells preincubated with calphostin C and treated with 1,25(OH)₂D₃. Outlined areas in Fig. 8 were analyzed for pixel intensity using Adobe Photoshop 6.0. Values for apical (●) and basal (◆) intensities are expressed as means $\pm$ S.D. ($n=2$).

Acknowledgements

This work was supported by NRI-CSREES-USDA grant 2004–35206–14134 (IN), and the Utah Agricultural Experiment Station. Approved as journal paper no. 7638. A portion of these results were presented by TMS in partial fulfillment for the degree of Masters of Science. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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Received 26 January 2005

Accepted 27 January 2005

Made available online as an

Accepted Preprint 7 February 2005