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Role of PKCβ in Signal Transduction for the 1,25D_3-MARRS Receptor (ERp57/PDIA3) in Steroid Hormone-Stimulated Calcium Extrusion

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Abstract

Protein kinase C (PKC) is involved in the rapid 1,25$(OH)_2D_3$-mediated extrusion of calcium in chick intestinal epithelial cells. A previous report demonstrated that PKCα and PKCβ redistribution corresponded to the dose-response curve for calcium extrusion. We investigated the role of both PKCα and PKCβ in hormone-stimulated calcium extrusion by using siRNA in primary cultures of intestinal cells. The results indicated that there was no change in calcium extrusion in cells transfected with siRNA to PKCα, whereas the siRNA to PKCβ significantly decreased calcium extrusion in 1,25$(OH)_2D_3$-treated cells when compared with the corresponding hormone-treated cells in transfected and nontransfected cells ($P < 0.01$). Similar results were also found using chemical inhibitors of PKCα (safingol) and PKCβ ([3-(1-(3-Imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione]) in intestinal cell suspension. The results demonstrated that PKCβ inhibited the extrusion of calcium (resulting in increased calcium uptake) from intestinal cells treated with 1,25$(OH)_2D_3$ when compared with vehicle controls ($P < 0.001$), the PKCα blocker did not show any difference in calcium extrusion among the treatment groups. Using confocal microscopy, we reduced the hormone exposure to 30 sec in order to view redistribution of PKCα and PKCβ. Rapid redistribution of PKCα was found to significantly increase in fluorescence in the apical membrane region after a 30 sec exposure of cells to 300- or 650 pM 1,25$(OH)_2D_3$ ($P < 0.01$ and 0.001, respectively). By comparison, PKCβ immunofluorescence was found to increase significantly in the basal lateral region of the cells, relative to controls after the exposure of cells to 300- ($P < 0.01$) or 650 pM ($P < 0.05$) seco-steroid. The results suggest that PKCα is the PKC isotype involved in 1,25$(OH)_2D_3$-mediated calcium efflux in intestinal epithelial cells.

Keywords: 1,25-dihydroxyvitamin D; rapid effects of steroid hormones; calcium uptake; intestinal cells

Introduction

Calcium, the most abundant mineral in vertebrates is an essential ion. It plays an important role in a variety of physiological functions including structural (skeletal and teeth) and signaling functions (muscle contraction, nerve impulse conduction, hormone and enzyme secretion, and as a secondary messenger in several pathways). Therefore, the control of calcium concentrations in the blood and extracellular fluid is necessary to maintain the normal functions of the cells as well as the prevention of calcium accumulation in the cells [8,31]. Normally, the input and output of calcium is adjusted to the physiological needs of the body. Either in normal or disturbed calcium homeostasis, cells will have a way to control events to protect them from cytotoxicity [12,13]. 1,25$(OH)_2D_3$ is one of the active metabolites if vitamin D and has been reported to maintain calcium homeostasis [4,12,13,24] and it has been suggested that PKC signal transduction is involved and mediated through the 1,25D_3-MARRS receptor/ERp57/PDIA3 [22].

A previous report demonstrated that PKC signaling has been identified in calcium extrusion [19] possibly by facilitating exocytosis of vesicular transport calcium [28], whereas the PKA signaling pathway is the likely mediator of calcium uptake in intestinal cells [12,13,26,29]. Studies in rat duodenum [2] have shown that 1,25$(OH)_2D_3$ can activate subcellular distribution of specific PKC isozymes, and hormone regulated enzyme modulation and redistribution were impaired with aging. In addition, Dranitzki-Elhalel et al. [5] suggested that the action of 1,25$(OH)_2D_3$ on calcium flux from cultured bone is mediated, in part, via activation of PKC. The study of Farhadi et al. [6] indicated that PKC normalized intracellular calcium by modulating calcium efflux. Korchak et al. [15] demonstrated that PKC is the regulator at several points in Ca^{2+} mobilization in neutrophils and HL60 cells. In addition, the Ca^{2+} dependent PKCs are known to regulate the activity of several membrane bound ion channels and carriers in various tissues, thereby modulating transmembrane ion transport [9], bicarbonate transport in rat jejunum [23], and Ca^{2+} pump and Ca^{2+} channel activity in sickle cells [7].

In small intestine, various PKC isoforms have been identified at the mRNA or protein level, including classical PKCs (cPKCs; [6]). Different isotypes elicit the differences in tissue expression, subcellular distribution, cofactor requirements and substrate specificity [10], and then lead each PKC to have a specific function in the signal transduction mechanism. However, how certain PKC isotypes influence the activation of calcium transport [2,16] in the small intestine remains unknown. The purpose of this study is to determine which Ca^{2+} dependent PKCs regulate the calcium efflux system, in order to provide a more knowledgeable cellular and molecular mechanism of 1,25$(OH)_2D_3$-stimulated calcium extrusion.

Materials and Methods

Animal and surgical methods

All protocols were approved by the Institutional Animal Care and

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Use 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 wks on a vitamin D-replete diet (Nutrena Feeds; Murray, UT). On the day of the experiments, chicks were anesthetized with chloropent (0.3 ml/100 g body weight), the duodenal loop was removed to ice-cold 0.9% saline and chilled for 15 min. The pancreas was excised and the duodenal loop was everted and rinsed three times with ice-cold saline.

Cell isolation

Intestinal epithelial cells were isolated as previously described [20,28]. The everted duodenal loop was gently stirred for 15 min at 23°C in citrate chelation solution containing 96 mM NaCl, 27 mM NaCitrate.2H_2O, 1.5 mM KCl, 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, pH 5.0. The intestinal segment was then transferred to fresh solution and cells released in the previous incubation transferred to 50-ml conical centrifuge tubes. The isolation protocol was repeated two more times. The cells were collected by low speed centrifugation (500 x g, 5 min, 4°C). Cell pellets were resuspended in Gey's Balanced Salt Solution (GBSS, 119 mM NaCl, 4.96 mM KCl, 0.22 mM KH_2PO_4, 0.84 mM $NaHPO_4$, 1.03 mM $MgCl_2.6H_2O$, 0.28 mM $MgSO_4.7H_2O$, 0.9 mM $CaCl_2$, pH 7.4). To minimize clumping, the cells were gently spread along the walls of the centrifuge tube with a Teflon rod, and room-temperature GBSS added drop-wise, with stirring, until a suspension was formed.

Cell suspensions and calcium uptake

Chicken intestinal cells were isolated by citrate chelation as described above and resuspended in room temperature Gey's Balanced Salt Solution (GBSS; 23°C) containing 0.1% BSA. Fourteen milliliters of cell suspension are pipetted into polypropylene tube which contained 1 μCi/ml of ^{45}CaCl2, final concentration (Perkin Elmer Life Sciences, Boston, MA), at T = -10 min. Aliquots (3.2 ml) of cell suspensions were pipetted into four fresh tubes and samples (100 μl) removed at T = -5 and -1 min to establish basal uptake. At T = 0 min, the cells were treated with vehicle ethanol (final concentration < 0.05%) or 300 pM 1,25$(OH)_2D_3$ to produce the following incubation conditions: (1) controls (2) 300 pM 1,25$(OH)_2D_3$ (3) control plus 80 μM PKCα blocker (safingol, EMD Chemicals, Gibbstown, NJ) (4) 300 pM 1,25$(OH)_2D_3$ plus 80 μM PKCβ blocker. Another series of experiments were done with the same procedures to produce the following conditions: (1) controls (2) 300 pM 1,25$(OH)_2D_3$ (3) control plus 30 nM PKCβ blocker [(3-(1-(3-imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione, EMD Chemicals, Gibbstown, NJ] (4) 300 pM 1,25$(OH)_2D_3$ plus 30 nM PKCβ blocker.

At T = 1, 3, 5, 7, and 10 min, 100 μl samples were pipetted into 900 μl of ice-cold GBSS to stop uptake. Cell samples were placed on ice and centrifuged (1,000 x g, 5 min, 4°C) at the end of the time course. The supernatants were decanted and the tubes swabbed with a Kimwipe while still in the inverted position. The pellets were resuspended in 500 μl of double-distilled water. The aliquots (100 μl) were pipetted into 3 ml of liquid scintillation cocktail for the determination of radioactivity and 20 μl of aliquots taken to determine protein using the Bradford reagent. Specific radioactivity (cpm/μg protein) was calculated and then values obtained for the treated phase normalized to average basal phase.

Cell culture

For studies with siRNA against PKCα or PKCβ (Ambion, Austin, TX, catalog numbers 4390771 and 4331182), cells were resuspended in 40 ml of GBSS and aliquots (0.2 ml) pipetted into 35 mm plastic Petri dishes (Falcon, Fisher Scientific; Franklin Lakes, NJ) with 3 ml of RPMI-1640 medium (Hyclone, Logan, UT), and antibiotics containing 100 units/ml of penicillin and 100 μg/ml of streptomycin (Sigma Chemical Co; St. Louis, MO). The cells were incubated overnight at 37°C, 5% CO_2/95% air without serum to promote cell adherence. The following day, medium was aspirated and the cells were transfected with SiImporter (Millipore, Bedford, MA) as described by the manufacturer's product protocol (Upstate, Lake Placid, NY, 2005) with 100 nM siRNA, final concentration (Ambion). Briefly, for each plate 930 μl of RPMI-1640 medium was combined with 65 μl of a solution containing 5 μl of SiImporter, 25 μl of siRNA diluent, 35 μl of RPMI-1640 and 5 μl of siRNA (transfected cells) or scrambled RNA (Santa Cruz Biotechnology, Santa Cruz, CA) or without siRNA (mock transfected cells), mixed gently and incubated at room temperature for 5 min. The transfection mixture was then added to the cultured cells and incubated for 4 hrs. After the incubation period, 3 ml of RPMI-1640 with 10% FBS and antibiotics were added to each Petri dish (Falcon Scientific Products; Franklin Lakes, NJ). Incubation was continued for 24 hrs at 37°C, 5% CO_2/95% air and the cultures were assayed for calcium uptake as described below.

Calcium uptake

After 48 hours of incubation, the medium was aspirated and replaced with 1 ml of GBSS containing 0.1% BSA (23°C). At T = 0, an equal volume of buffer was added containing radioactivity (1 μCi of ^{45}CaCl2 [Perkin Elmer Life Sciences, Boston, MA] per ml, final concentration) and either vehicle (0.012% ethanol, final concentration) or 300 pM 1,25$(OH)_2D_3$ (final concentration), and the incubation continued for 7 min—a time found earlier to be optimal for observing hormone-mediated differences in uptake [25]. Media were then aspirated and the cells were rinsed with 4 ml of ice-cold GBSS three times, followed by the addition of 0.5 ml of lysis buffer (0.1% (v/v) triton X-100 in TED containing 10 mM Tris, 1.25 mM EDTA, 2mM dithiothreitol, pH 7.4). Cells were scraped, solubilized and collected for determination of radioactivity by liquid scintillation spectrophotometry and protein. The remaining samples were frozen for Western analyses.

Confocal microscopy

Intestinal cells were isolated by citrate chelation and resuspended in 40 ml of GBSS. A coverslip was placed in the bottom of each Petri dish and 3 ml of RPMI 1640 and 0.5 ml of cell suspension added. The cells were cultured overnight. The following morning, media were replaced with 1 ml of GBSS containing 0.1% BSA. At T = 0 min, either vehicle (0.001% ethanol, final concentration), 130-, 300-, or 650 pM 1,25$(OH)_2D_3$ were added. At T = 30 sec, media were replaced with 1 ml of 3% paraformaldehyde, 3% sucrose in PBS and the cells are fixed for 20 min. After washing three times with PBS, cells were permeabilized for 5 min with 0.1% Triton X-100 in PBS, washed three times with PBS, and incubated with primary antibody (rabbit anti PKCα or mouse anti PKCβ, 1/500 dilution with PBS-0.1% BSA) for 30 min, followed by three washes with PBS. Cells were then incubated with fluorescein-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA) for 30 min, washed three times and mounted on 10% 1 M TRIS in 80% glycerol on a microscope slide. Coverslips were blotted, and sealed with nail polish. Prepared samples were analyzed using a BioRad MRC 1024 laser-scanning confocal microscope system mounted in the Keller position and attached to a Nikon TE-200 microscope. Images were captured with BioRad LASERSHARP acquisition software, using

a 40x objective lens [28]. The images were analyzed for pixel intensity using Adobe Photoshop 6.0 (Adobe Systems, Inc., San Jose, CA).

Protein determination

The protein concentration was determined using Bradford reagent (BioRad) and bovine γ-globulin as the protein standard.

Statistical analysis

All values were expressed as mean ± SEM. Statistical comparisons between two treatment groups were compared by one way ANOVA (SigmaStat 3.1 and SigmaPlot 9.0). Significant differences were considered to occur at $P < 0.05$.

Results

Effect of siRNA against PKCα on calcium extrusion in cultured intestinal cells

We investigated the effect of knockdown of PKCα gene expression on calcium efflux. In this study, we cultured intestinal cells as previously described [20,28], and transfected intestinal primary cell cultures with siRNA against PKCalpha. Cells were treated with vehicle control or 300 pM steroid hormone. The result was that no difference was observed between nontransfected and transfected cells (Figure 1).

Effect of siRNA against PKCβ on calcium extrusion in cultured intestinal cells

Under the same conditions as the previous experiments, siRNA against PKCβ resulted in a significant increase in calcium uptake in hormone treated (169.39 + 3.55^{aaa}, P < 0.001), relative to nontransfected controls and hormone treated (82.66 + 2.06, 144.05 + 4.03, respectively) and mock transfected cells (79.59 + 2.30). No difference was found in nontransfected and cells transfected with scrambled siRNA (Figure 2). Western blots with anti-PKCα and PKCβ verified the protein expression was decreased approximately by 40% (data not shown). We were subsequently made aware that the siRNAs might not be completely specific for a given isotype. So while suggestive, we chose to pursue analysis of the roles of the isotypes using chemical blockers.

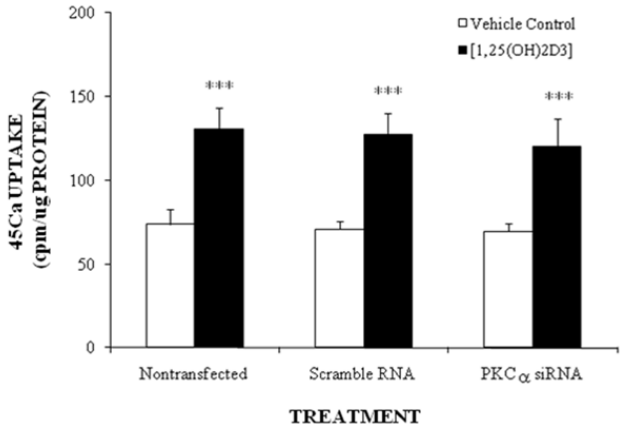

Figure 1: Effect of siRNA to PKCα on calcium uptake in cultured intestinal cells. Isolated cells were resuspended in 40 ml of GBSS (Gey's balanced salt solution) and 200 µl pipetted into 35 mm plastic Petri dishes containing 3 ml of RPMI-1640 medium and antibiotics (100 units/ml penicillin, 100 mg/ml streptomycin, Sigma). The cells were incubated for 24 h (in the absence of serum) at 37°C with 5% CO_2/95% air to promote cell adhesion and then transfected. The transfection protocol is described in material and methods. After 24 h cultured cells were incubated with or without 300 pM 1,25$(OH)_2D_3$ in 1 ml of GBSS/0.1% (wt/vol) BSA containing 4 µCi of $^{45}CaCl_2$ for 7 min. The cells were rinsed 3 times with 4 ml ice-cold GBSS, followed by the addition of 0.5 ml of 0.1% (vol/vol) Triton X-100 in 10 mM TED to each plate. Cells were scraped and radioactivity determined by liquid scintillation counting. Data are represented as mean ± S.E.M. (***$P < 0.001$, relative to corresponding controls).

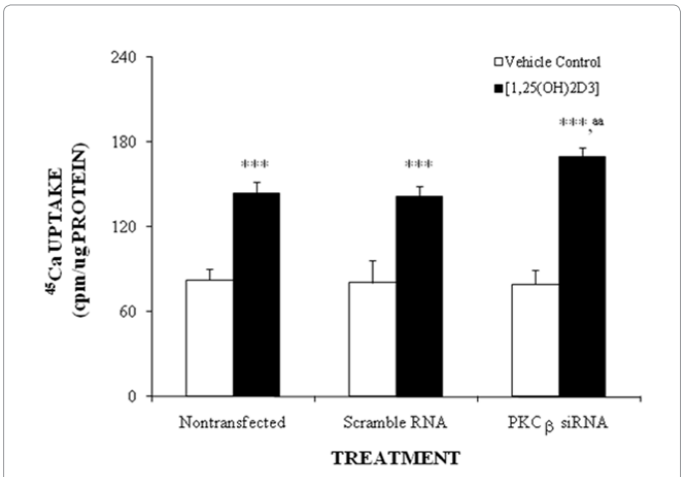

Figure 2: Effect of siRNA to PKCβ on calcium uptake in cultured intestinal cells. Cells were cultured and treated as described in Fig. 1. Data are represented as mean + S.E.M. (aa, ***$P < 0.01$, 0.001, relative to corresponding hormone-treated and vehicle controls, respectively).

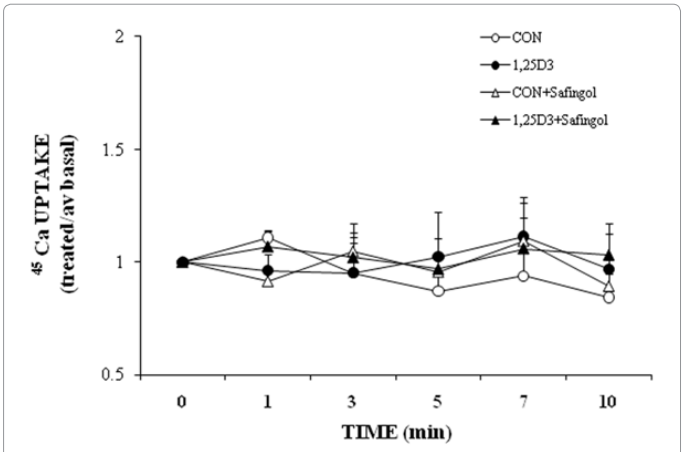

Figure 3: Effect of the chemical PKCα blocker (safingol) on calcium uptake in intestinal cell suspensions. Enterocytes isolated by citrate chelation were resuspended in Gey's Balanced Salt Solution (GBSS; 23°C) containing 0.1% BSA and combined with 2 µCi/ml of $H_3{}^{32}PO_4$, final concentration, at the start of the time course studies. Aliquots of cells were pipetted into four fresh tubes and samples (100 µl) were removed at T = -5 and -1 min to establish basal uptake. At T = 0 min, the cells were treated with the vehicle ethanol (final concentration < 0.05%) or 300 pM 1,25$(OH)_2D_3$ to produce the following incubation conditions: (1) controls (open circles) (2) 300 pM 1,25$(OH)_2D_3$ (closed circles) (3) controls in the presence of the PKCα blocker (open triangles) (4) 300 pM 1,25$(OH)_2D_3$ in the presence of the PKCα blocker (closed triangles). Data are presented as mean treated/average basal ratios ± SEM.

Effect of PKCα blocker (safingol) on 1,25$(OH)_2D_3$-mediated calcium extrusion

To determine whether PKCα mediates calcium extrusion, freshly isolated intestinal cells were treated with vehicle or 300 pM 1,25$(OH)_2D_3$ in the absence or presence of inhibitor, and aliquots removed at the indicated times. The results from experiments using the chemical PKCα blocker demonstrated no difference in calcium extrusion (lack

of altered calcium uptake) in vehicle controls, $1,25(OH)_2D_3$ treated, vehicle control plus PKCα blocker or $1,25(OH)_2D_3$ plus PKCα blocker groups (Figure 3). Therefore, it is likely that PKCα is not the PKC isotype involved in steroid hormone-stimulated calcium efflux from intestinal epithelial cells. The lack of an effect of $1,25(OH)_2D_3$ on freshly isolated intestinal cells (Figure 3) was first reported by Nemere and Campbell [19]. Since calcium transport in perfused duodena was stimulated by the steroid hormone [16], we reasoned that both uptake and efflux were stimulated to a similar extent, therefore no visible effects occurred in isolated intestinal cells.

Effect of PKCβ blocker [(3-(1-(3-imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione] on $1,25(OH)_2D_3$-mediated calcium extrusion

The equivalent experiments were undertaken to study the involvement of PKCβ on calcium efflux. The effect of the chemical PKCβ inhibitor was to cause a significant decrease in calcium extrusion (increase in calcium uptake) in cells treated with 300 pM $1,25(OH)_2D_3$ plus PKCβ blocker at 3, 5, 7 and 10 min ($1.311 + 0.086^{bb}$, $1.396 + 0.168^{bbb}$, $1.454 + 0.160^{bb}$ and $1.400 + 0.067^{bb}$, respectively, $^{bb,bbb}P < 0.01$, 0.001) when compared with $1,25(OH)_2D_3$ at the corresponding time points (1.025 + 0.062, 0.993 + 0.087, 1.001 + 0.094 and 0.963 + 0.096, respectively; Figure 4). The percentage decrease in calcium efflux was approximately 29% (3 min), 40% (5 min), 45% (7 min) and 44% (10 min) when compared to cells treated with PKCβ blocker plus $1,25(OH)_2D_3$ and cells treated with hormone- alone.

Effect of $1,25(OH)_2D_3$ on anti-PKCα immunofluorescence.

We used confocal microscopy to determine the involvement of PKCα in steroid hormone-mediated signal transduction. Intestinal cells were cultured on cover slips, treated with vehicle or 130-, 300-, or 650 pM $1,25(OH)_2D_3$ for 30 sec in order to assess the redistribution of PKCα. Cultured cells were incubated with anti-PKCα and the brush border membranes were stained with rhodamine-conjugated phalloidin. Non specific staining controls were negative (data not shown). Figure 5A demonstrates the distribution of fluorescein conjugated secondary antibody to anti- PKCα which was found to be greater in apical part of intestinal cells treated with 300- and 650 pM ($P < 0.01$, 0.001, respectively) than in controls or in the 130 pM treatment group. The graph shown in (Figure 5B) depicts the results of confocal microscopy pixel intensity with the data were corrected for non specific controls. The percentage increase in pixel intensity was 21% and 25% in apical region of the cells and 15% and 26% in brush border plus apical area in 300- and 650 pM steroid-treated cells, respectively, when compared with the vehicle controls. There is no difference in pixel intensity in brush borders or basal lateral membranes alone. This result showed that there was the rapid redistribution of PKCα when the cells were treated with hormone.

Effect of $1,25(OH)_2D_3$ on anti-PKC immunofluorescence distribution as judged by confocal microscopy

Figure 6A shows the distribution of fluorescein-conjugated

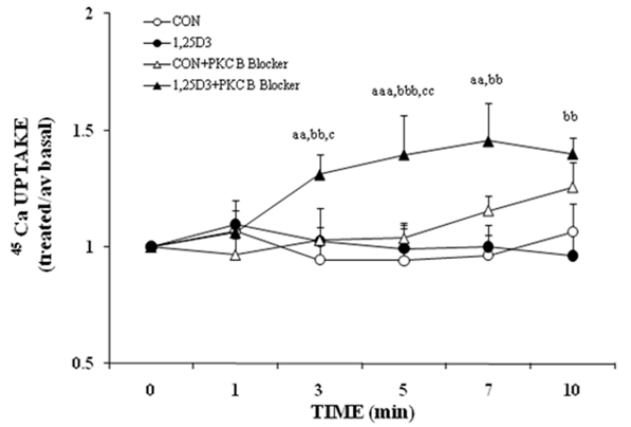

Figure 4: Effect of the chemical PKCβ blocker ([3-(1-(3-Imidazol-1-ylpropyl)-1H-indol-3-yl)-4-anilino-1H-pyrrole-2,5-dione]) on calcium uptake in intestinal cells suspension. Another series of experiments were done with the same procedure as described in Fig 3 to produce the following conditions: (1) controls (open circle) (2) 300 pM $1,25(OH)_2D_3$ (closed circles) (3) controls in the presence of the PKCβ blocker (open triangles) (4) 300 pM $1,25(OH)_2D_3$ in the presence of the PKCβ blocker (closed triangles). At T = 1, 3, 5, 7, and 10 min, 100 µl samples were pipetted into 900 µl of ice-cold GBSS. Cell samples were centrifuged (1,000 x g, 5 min, 4°C), supernatants decanted and pellets analyzed for radioactivity and protein. Specific radioactivity (cpm/µg protein) was calculated and then values obtained for the treated phase were normalized to average basal phase. Data are presented as mean treated/average basal ratios ± SEM. (aa,bb,cc $P < 0.01$ and $^{aaa,bbb,ddd}P < 0.001$, relative to controls, $1,25(OH)_2D_3$ and controls plus PKCβ blockers, respectively).

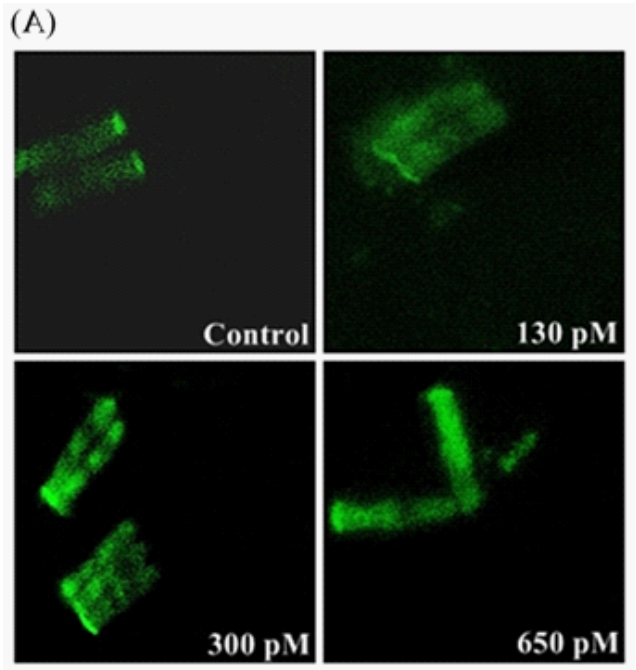

Figure 5A: Immunocytochemical localization of PKCα in isolated intestinal cells. Intestinal cells were isolated by citrate chelation, resuspended in 40 ml of GBSS and cultured with 3 ml of RPMI 1640 in tissue culture dishes containing a cover slip to which were added 0.5 ml of cell suspension. The cells were cultured overnight. The following morning, media were aspirated and replaced with 1 ml of GBSS containing 0.1% BSA. At T = 0 min, either vehicle (0.001% ethanol, final concentration), 130-, 300-, or 650 pM $1,25(OH)_2D_3$ were added. At T = 30 sec, media were aspirated and replaced with 1 ml of 3% paraformaldehyde, 3% sucrose in PBS and the cells are fixed for 20 min. After washing three times with PBS, cells were permeabilized for 5 min with 0.1% Triton X-100 in PBS, washed three times with PBS, and incubated with primary antibody (rabbit anti PKCα, 1/500 dilution) with PBS-0.1% BSA for 30 min, followed by three washes with PBS. Cells were then incubated with fluorescein-conjugated secondary antibody (Jackson Immunoresearch, West Grove, PA) for 30 min, washed three times and mounted on 10% 1 M TRIS in 80% glycerol on microscope slide. Coverslip were blotted, and sealed with nail polish. Arrows indicate the brush border.

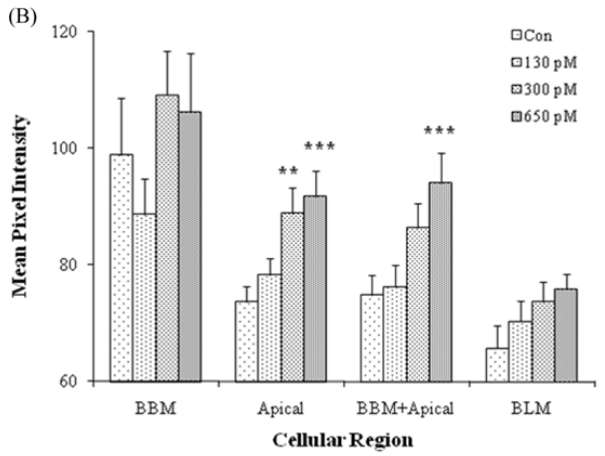

Figure 5B: The graph illustrates mean pixel intensity of fluorescein-conjugated secondary against anti-PKCα in different cellular regions. Data represent mean ± SEM (**, *** P < 0.01, 0.001 when compared with the corresponding controls).

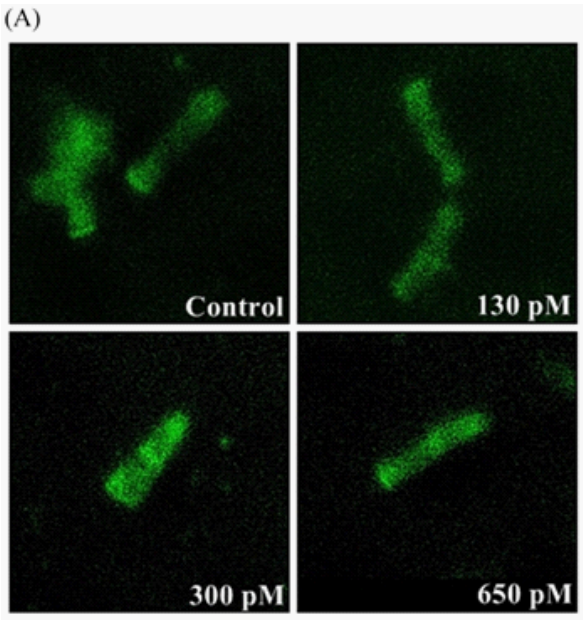

Figure 6A: Immunocytochemical localization of PKCbeta in isolated intestinal cells. Cells were prepared and treated as described in Figure 5 (A), except the cells were incubated with mouse anti PKCβ, 1/500 dilution as the primary antibody. Arrows indicate the brush border.

secondary antibody to anti- PKCβ. In contrast to the distribution of anti-PKCα, the anti-PKCβ fluorescein was found to be greater in the basal lateral region of intestinal cells treated with 300- and 650 pM (P < 0.01, 0.05, respectively) when compared with controls and the 130 pM treatment group. Figure 6B presents these results in graphic form in which pixel intensity was corrected for non specific controls. The percentage increase in pixel intensity was 18% and 13% in the basal lateral region of cells treated with 300- and 650 pM, respectively, when compared with vehicle controls. There were no differences in pixel intensity in other cellular regions. These results demonstrate that PKCβ was redistributed after incubating cells with 1,25$(OH)_2D_3$.

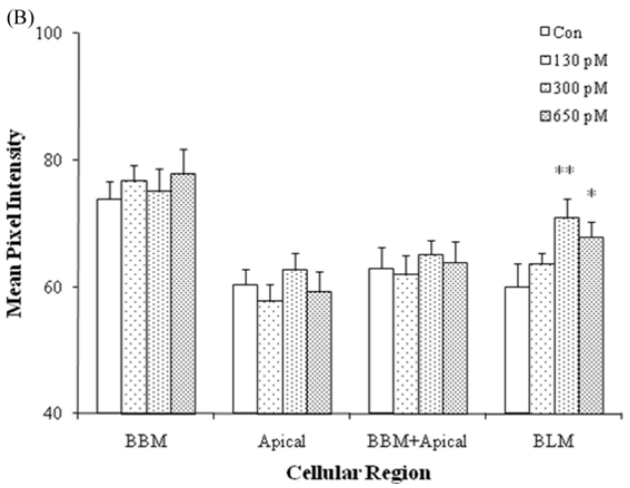

Figure 6B: Quantitative analysis of the distribution of fluorescence intensity. The graph presents the mean pixel intensity of anti-PKCβ fluorescence at different cellular regions. Data are presented as mean ± SEM (*,** P < 0.05, 0.01 when compared with corresponding controls).

Discussion

Using Forskolin, a PKA activator, we have reported elsewhere that the PKA signaling pathway is the mediator of calcium uptake in intestinal cells [12,13,26,28], whereas the study by Nemere and Campbell [19] has identified that PKC signaling is involved in calcium extrusion. A recent study [13] has reported that phorbol ester, a PKC activator, stimulated Ca^{2+} efflux, and forskolin stimulated uptake. Furthermore, our previous study [30] showed the dose response curve of 1,25$(OH)_2D_3$-stimulated PKCβ activity exhibited activation at both 300- and 650 pM hormone, which corresponds with the curve for activated calcium transport [21].

In the current study, we used chemical blockers for PKCα and PKCβ and found that use of the PKCβ blocker resulted in stimulated calcium uptake in intestinal cells treated with the 1,25$(OH)_2D_3$. The result indicates that the increase in calcium uptake, resulting from an inhibition of calcium efflux from the cells was due to the 1,25$(OH)_2D_3$ mediated PKCβ signal transduction pathway. While equivalent experiments were performed to identify the involvement of PKCα, the results showed there was no difference in calcium uptake in cells treated with the PKCα blocker plus steroid hormone when compared to the vehicle control and hormone treated cells alone. From this result, it is likely to conclude that PKCβ in not involved in 1,25$(OH)_2D_3$-mediated calcium extrusion.

Correspondingly, the experiments with siRNA against PKCβ in primary intestinal cell culture showed an increase in calcium uptake (in turn, decreased calcium extrusion) of 25% when compared with cells treated with 1,25$(OH)_2D_3$. This result is lower than with the chemical inhibitor and can be explained by the loss of PKC activity after 72 h of incubation in cell culture [13]. In comparison, siRNA against PKCα demonstrated no difference in calcium uptake in all treatment groups of both transfected and nontransfected cells. From the chemical blockers and transfection studies, our findings clearly indicate that PKCβ is the PKC isotype that mediates the 1,25D_3-MARRS receptor-stimulated calcium extrusion in intestinal epithelial cells whereas PKCα and PKCβ are involved in steroid-enhanced phosphate uptake [30].

Our study was similar to the study of Farhadi et al. [6] in Caco-

2 cells. They reported that PKCβ protects the intestinal cell from oxidant-induced injury through PKC normalized intracellular calcium by modulating calcium efflux. By contrast, Fathallah et al. [7] have studied the effect of PKC in deoxygenated sickle cells and suggested that PKCβ stimulated Ca^{2+} efflux by mediating the phosphorylation of a Ca^{2+} pump or an associated protein. In addition, Korchak et al. [15] has report that PKCβ regulates ligand-initiated Ca^{2+} uptake in differentiated HL60 cells.

By using confocal microscopy, we have identified the translocation of PKCβ to the basal lateral region of intestinal cells whereas PKCα translocated to the apical part in cells treated with different concentration of steroid hormone within 30 sec. The difference in PKC translocation might be because these two PKC isotypes function differently in 1,25$(OH)_2D_3$-mediated ion transport since individual isotypes have distinct and specialized functions in cell signaling [10]. However, a similar result has been reported. Sitrin et al. [27], have demonstrated that 1,25$(OH)_2D_3$ rapidly stimulates the translocation of PKCβ, to the basolateral membrane, not the brush border membrane. However, they found that PKCα, did not translocate to either antipodal plasma membrane. Balogh et al. [2] also demonstrated that 1,25$(OH)_2D_3$ affects the subcellular distribution of PKCα, β and δ and these effects are impaired with aging. These results might be different because of the tissue specificity and species; we have studied chick intestinal epithelial cells whereas they studied rat colonic cells.

However, the exact mechanism involved in 1,25$(OH)_2D_3$ mediated calcium extrusion is still unknown. It might be that PKC stimulated vesicular transport of calcium [28] in either a receptor mediated or non receptor mediated pathway [1,17,18]. Alternatively, 1,25$(OH)_2D_3$ has been reported to upregulate plasma membrane Ca^{2+}-ATPase expression [14] and studies of PKC in many cell types have shown increased activity Ca^{2+}-ATPase and is activated by PKC [11]. In conclusion, we suggest that PKCβ mediates signal transduction for the 1,25D_3-MARRS receptor (ERp57/PDIA3) in steroid hormone-stimulated calcium extrusion in chick intestinal epithelial cells, possibly by the regulation of exocytosis through the basal lateral membrane and/or by activation of Ca^{2+}-ATPase on the basal lateral membrane of chick enterocytes.

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