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Contributions of Pro-Oxidant and Anti-Oxidant Conditions to the Actions of 24,25-Dihydroxyvitamin D_3 and 1,25-Dihydroxyvitamin D_3 on Phosphate Uptake in Intestinal Cells

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Abstract The steroid hormone 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] rapidly stimulates the uptake of phosphate in isolated chick intestinal cells, while the steroid 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$] inhibits the rapid stimulation by 1,25$(OH)_2D_3$. Earlier work in this laboratory has indicated that a cellular binding protein for 24,25$(OH)_2D_3$ is the enzyme catalase. Since binding resulted in decreased catalase activity and increased H_2O_2 production, studies were undertaken to determine if pro-oxidant conditions mimicked the inhibitory actions of 24,25$(OH)_2D_3$, and anti-oxidant conditions prevented the inhibitory actions of 24,25$(OH)_2D_3$. An antibody against the 24,25$(OH)_2D_3$ binding protein was found to neutralize the inhibitory effect of the steroid on 1,25$(OH)_2D_3$-mediated ^{32}P uptake. Incubation of cells in the presence of 50 nM catalase was also found to alleviate inhibition. In another series of experiments, isolated intestinal epithelial cells were incubated as controls or with 1,25$(OH)_2D_3$, each in the presence of the catalase inhibitor 3-amino-1,2,4-triazole, or with 1,25$(OH)_2D_3$ alone. Cells exposed to hormone alone again showed an increased accumulation of ^{32}P, while cells treated with catalase inhibitor and hormone had uptake levels that were indistinguishable from controls. We tested whether inactivation of protein kinase C (PKC), the signaling pathway for ^{32}P uptake, occurred. Incubation of cells with phorbol-13-myristate (PMA) increased ^{32}P uptake, while cells pretreated with 50 μM H_2O_2 prior to PMA did not exhibit increased uptake. Likewise, PMA significantly increased PKC activity while cells exposed to H_2O_2 prior to PMA did not. It is concluded that catalase has a central role in mediating rapid responses to steroid hormones. J. Cell. Biochem. 101: 1176–1184, 2007.

Key words: 1,25D_3-MARRS receptor; 24,25-dihydroxyvitamin D_3; catalase; reactive oxygen species; phosphate uptake; intestinal cells; ERP57/GR58/PDIA3

The vitamin D endocrine system is a key mediator of phosphate and calcium homeostasis. Regulation of new, dietary mineral uptake occurs in intestine, while in kidney reabsorption is modulated. Deposition of these minerals in bone is also under hormonal control. Regulation by vitamin D metabolites can occur through the classical vitamin D receptor (VDR) as well as cell surface receptors.

Activation of vitamin D begins in the liver with production of 25-hydroxyvitamin D_3, followed by renal hydroxylation to yield either 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] or 24,25-dihydroxyvitamin D_3 [24,25$(OH)_2D_3$]. While 1,25$(OH)_2D_3$ stimulates the rapid transport of calcium and phosphate, 24,25$(OH)_2D_3$ inhibits such stimulation (Nemere, 1996, 1999; Zhao and Nemere, 2002). Hormone-stimulated phosphate uptake in chick intestinal cells is initiated by ligand binding to the 1,25D_3-*m*embrane *a*ssociated, *r*apid *r*esponse *s*teroid-binding receptor (MARRS), which is equivalent to ERp57/GR58/PDIA3, and which contains two thioredoxin folds (Nemere et al., 2004). To elucidate how 24,25$(OH)_2D_3$ might effect inhibition, since it does not compete for binding the 1,25D_3-MARRS receptor [Nemere et al., 1994], we purified a cellular binding protein with $K_d = 7$ nM 24,25 $(OH)_2D_3$ [Nemere et al., 2002]. While specific

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binding was found at the cell surface [Watanabe et al., 2003], the preponderance of binding material was vesicular [Nemere et al., 2002]. Using the appropriate starting material, we purified and sequenced the $24,25(OH)_2D_3$ binding protein and subsequently identified it as catalase [Larsson et al., 2006], an enzyme previously reported to be sensitive to cell signaling molecules [Yano and Yano, 2002]. A functional consequence of $24,25(OH)_2D_3$ binding to catalase was found to be a decrease in enzyme activity, with concomitant increase in H_2O_2 production [Nemere et al., 2006]. One mechanism of inhibition was then found to be oxidation of the $1,25D_3$-MARRS receptor and inhibition of $[^3H]1,25(OH)_2D_3$ binding [Nemere et al., 2006]. We then established that antioxidant diets nearly doubled phosphate absorption in vivo [Nemere et al., 2006]. The current study was undertaken to investigate the effects of selected reducing and oxidizing states on phosphate uptake at the cellular level. Our hypothesis is that antioxidant conditions—such as an anti-catalase antibody or excess exogenous catalase protein—will block the inhibitory actions of $24,25(OH)_2D_3$, whereas pro-oxidant conditions—such as a triazole inhibitor of catalase activity, will mimic the inhibitory actions of the secosteroid.

MATERIALS AND METHODS

Preparation of Ab 365

This was previously described by Larsson et al. [2006]. The $24,25(OH)_2D_3$ binding protein was isolated from the P_2 fraction (20,000*g*, 10 min, post-nuclear pellet) by a combination of anion exchange-and gel filtration chromatography on the basis of enriched binding of $[^3H]24,25(OH)_2D_3$. The 64-kDa fraction was resolved on an 8% SDS–polyacrylamide gel, stained with Coomassie Brilliant Blue, and excised for tryptic digestion and sequencing of the resulting peptides [Larsson et al., 2006]. Equivalent protein was used to immunize rabbits, and the resulting antisera shown to react with commercially available catalase [Larsson et al., 2006].

Animals and Surgical Procedures

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

Isolation of Epithelial Cells

Intestinal epithelial cells were isolated by the citrate chelation method. The duodenal loop was transferred to 30 ml of Solution A containing 96 mM NaCl, 1.5 mM KCl, 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, 27 mM sodium citrate, pH 5.0 [Nemere et al., 2004; Sterling and Nemere, 2005], and stirred for 15 min to release the cells. The process was repeated twice with fresh Solution A. The cells were collected from the pooled isolation media by centrifugation at 500*g* for 5 min at 4°C. After decanting the supernatant, and while still in the inverted position, the inside of the tube was swabbed with a Kimwipe.

The intestinal cells were resuspended in 20 ml of Gey's Balanced Salt Solution (GBSS) lacking bicarbonate [Sterling and Nemere, 2005] and containing 119 mM NaCl, 4.96 mM KCl, 0.22 mM KH_2PO_4, 1.03 mM $MgCl_2.6H_2O$, 0.28 mM $MgSO_4$, 0. 9 mM $CaCl_2$, by gentle dispersion with a Teflon-coated rod to avoid breaking the fragile intestinal cells, followed by drop wise addition of GBSS.

^{32}P Uptake and Ab 365 to the $24,25(OH)_2D_3$ Binding Protein

Fourteen milliliters of the cell suspension in GBSS was removed to a polypropylene tube (Falcon, Fischer Scientific, Dallas, TX) containing 28 μCi of $H_3{}^{32}PO_4$ (Perkin Elmer Life Sciences, Boston MA), thereby initiating the time course. Aliquots (3.2 ml) of cell suspension were then pipetted into four fresh polypropylene tubes, two of which contained Ab 365 (1/100 dilution, final concentration) against the $24,25(OH)_2D_3$ binding protein. Aliquots (100 μl) were removed at $T = -5$ min and -1 min to establish basal uptake rates of ^{32}P. At $T = 0$ min,

the cells were treated with the vehicle ethanol (<0.05% final concentration) or steroids to give the following incubation conditions: (1) controls plus Ab 365; (2) 300 pM $1,25(OH)_2D_3$; (3) 300 pM $1,25(OH)_2D_3$ plus 6.5 nM $24,25(OH)_2D_3$; or (4) 300 pM $1,25(OH)_2D_3$ plus 6.5 nM $24,25(OH)_2D_3$ plus antibody Ab 365. At T = 1, 3, 5, 7, and 10 min, 100 μl samples were taken. Each 100 μl sample was pipetted into 900 μl of ice cold GBSS to stop uptake and dilute radionuclide.

The samples were held on ice and centrifuged at the end of the time course for 5 min at 1,000*g* at 4°C. Supernatants were decanted and while still in the inverted position, the insides of the tubes were swabbed. The pellets were resuspended in 500 μl of double-distilled water, and 100 μl aliquots taken for liquid scintillation spectrophotometry and 20 μl for protein determination. For each sample in the treated phase, cpm/μg of protein was normalized to the average basal cpm/μg of protein. Protein was assessed using the Bradford reagent (BioRad, Hercules, CA) against bovine γ-globulin as standard.

^{32}P Uptake and Exogenous Catalase

In another series of experiments, cell suspension plus radionuclide was pipetted into four fresh tubes, two of which contained bovine catalase (Sigma Chemical Co, St. Louis, MO) with subsequent addition of vehicle or steroids. The treatement conditions were (1) vehicle controls; (2) 300 pM $1,25(OH)_2D_3$ plus 6.5 nM $24,25(OH)_2D_3$; (3) 300 pM $1,25(OH)_2D_3$ plus 6.5 nM $24,25(OH)_2D_3$ plus 50 nM catalase; (4) 300 pM $1,25(OH)_2D_3$ plus 6.5 nM $24,25(OH)_2D_3$ plus 1 μM catalase.

^{32}P Uptake and Catalase Inhibitor

Cell suspensions were combined with $H_3{}^{32}PO_4$ as described above and aliquots pipetted into fresh tubes, two of which contained 1 μM 3-amino-1.2.4-triazole (final concentration, Sigma). At T = 0, suspensions were treated with vehicle or steroid. The treatment groups were (1) controls plus inhibitor; (2) 300 pM $1,25(OH)_3D_3$; and (3) 300 pM $1,25(OH)_3D_3$ plus catalase inhibitor.

Determination of PKC Activity

Enzyme activity was determined in cell suspensions treated with vehicle, 300 pM $1,25(OH)_2D_3$, 100 nM phorbol myristate acetate (Sigma), or phorbol in cells pretreated with 50 μM H_2O_2. Cell suspensions were sampled at T = 0, 1, 3, and 5 min. PKC activity was analyzed using a commercially available assay system (Upstate, Lake Placid, NY). After incubation, as described above, the cells were centrifuged at 1,000*g* at 4°C for 10 min, the supernatant was decanted, and the pellet was stored at −20°C until analysis could be performed. Samples were placed on ice, 500 μl of chilled double-distilled water was added, and the samples homogenized. Ten microliters of each sample (containing 10–200 μg protein) were transferred into microfuge tubes on ice. Enzyme activity was assayed using [γ ^{32}P] ATP, and the substrate peptide [QKRPSQRSKYL]. Aliquots of the incubation mixtures were spotted onto phosphocellulose disks; the disks were washed and placed in scintillation vials for assessment of incorporated radioactivity.

RESULTS

^{32}P Uptake and Ab 365 to the $24,25(OH)_2D_3$ Binding Protein

Figure 1 shows the results of experiments designed to test whether neutralization of the $24,25(OH)_2D_3$ binding protein (catalase) affects inhibition of the $1,25(OH)_2D_3$ response. Vehicle controls (final concentration 0.05% ethanol) incubated in the presence of Ab 365 showed a general decrease in ^{32}P levels with time (open circles), as previously reported [Zhao and Nemere, 2002]. However, the extent of the decrease was not influenced by the presence of antibody, as can be seen by comparison with controls in subsequent figures. Addition of $1,25(OH)_2D_3$ to cell suspensions incubated in parallel resulted in enhanced levels of ^{32}P uptake that were evident within 1 min of hormone addition (Fig. 1), and which became statistically significant at 3 to 10 min ($P < 0.001$), relative to corresponding controls. At 10 min of incubation, $1,25(OH)_2D_3$-treated cells accumulated radionuclide to 132% of controls. Incubation of cells in the presence of both $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ resulted in ^{32}P uptake levels that were not significantly different than controls (Fig. 1), also as previously reported [Zhao and Nemere, 2002]. However, pre-incubation of cells with Ab 365 and both steroids reversed the inhibition (Fig. 1). Radionuclide levels were noticeable greater in such cell suspensions within 1 min, and became significantly greater than

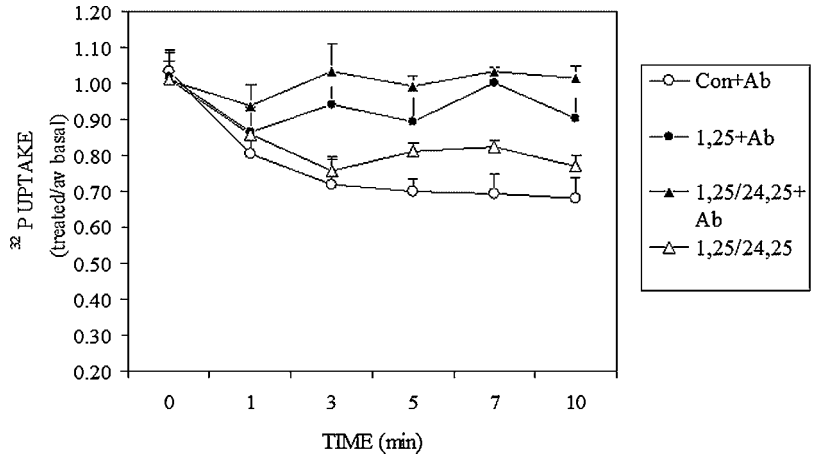

Fig. 1. Time course of ^{32}P Uptake and effect of Ab 365 against the $24,25(OH)_2D_3$ binding protein. Enterocytes isolated by citrate chelation were resuspended in Gey's Balanced Salt Solution (GBSS; 23°C), and combined with 2 μCi/ml of $H_3{}^{32}PO_4$, at which point the time course was initiated. Aliquots of cell suspension were pipetted into four fresh tubes, three of which contained Ab 365 at a 1/100 final dilution. Samples were removed at T = −5 and −1 min to establish basal uptake. At T = 0 min, the cells were treated with vehicle or steroids to produce the following incubation conditions: (1) controls plus Ab 365 (○–○; n = 7); (2) 300 pM $1,25(OH)_2D_3$ plus Ab 365 (●–●; n = 7); (3) 300 pM $1,25(OH)_2D_3$ plus 6.5 nM $24,25(OH)_2D_3$ (△–△; n = 7); (4) 300 pM $1,25(OH)_2D_3$ plus 6.5 nM $24,25(OH)_2D_3$ plus Ab 365 (▲–▲; n = 7). At T = 1, 3, 5, 7, and 10 min, 100 μl samples were taken and pipetted into 900 μl of ice-cold GBSS. Cell samples were centrifuged (1,000*g*, 5 min), supernatants decanted, and pellets analyzed for radioactivity and protein. Radionuclide was related to corresponding cellular protein in each aliquot, and then normalized to average basal cpm/μg protein. Data are presented as mean treated/average basal ratios ± SEM.

corresponding incubations without antibody at 3 to 10 min ($P < 0.001$) (Fig. 1) After 10 min of incubation, cells treated with both steroids in the presence of Ab 365 exhibited ^{32}P uptake that was 150% of cells incubated with both steroids and no antibody.

To determine whether antibody-induced neutralization occurred through inhibition of catalytic activity or inhibition of steroid binding, subcellular fractions enriched in these activities [Nemere et al., 2002; Larsson et al., 2006] were tested by incubation either with Ab 365—which recognizes catalase [Larsson et al., 2006] or the control Ab 377—which did not give a reaction product upon Western analysis (data not shown). Determination of catalase activity in Percoll gradient fractions 1 and 2 indicated incubation with Ab 365 yielded 98% and 106%, respectively, of control values. Analysis of specific binding of $[^3H]24,25(OH)_2D_3$ [Larsson et al., 2006] under parallel conditions indicated that in fractions exposed to Ab 365 levels were reduced to 61% and 64% of corresponding controls in fractions 1 and 2, respectively.

^{32}P Uptake and Effect of Exogenous Catalase

The results of experiments designed to test whether addition of exogenous catalase overcomes the inhibitory effect of $24,25(OH)_2D_3$ are shown in Figure 2. Vehicle controls showed a general decrease in ^{32}P levels with time (open circles), to a similar extent as observed in Figure 1. Cells exposed to a combination of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ also had uptake levels that were not significantly different than controls (Fig. 2), again as previously reported [Zhao and Nemere, 2002]. Exogenous catalase reversed the $24,25(OH)_2D_3$-mediated inhibition of $1,25(OH)_2D_3$. Addition of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ and either 0.05 or 1.0 μM bovine catalase to cell suspension incubated in parallel resulted in enhanced levels of ^{32}P uptake that were evident within 1 min of hormone addition, and which became statistically significant at 5 to 10 min ($P = 0.02$ to 0.001), relative to corresponding controls or corresponding samples from cells treated with both steroids alone. At 10 min of incubation, cells treated with both steroids plus 0.05 μM catalase accumulated radionuclide to 140% of vehicle controls, while those exposed to 1 μM catalase exhibited ^{32}P levels that were 135% of vehicle controls. In other studies, it was noted that a 33 nM catalase solution lost approximately 75% enzymatic activity within 5 min of dilution into buffer. This observation would suggest that alleviation of inhibition by exogenous catalase was a result

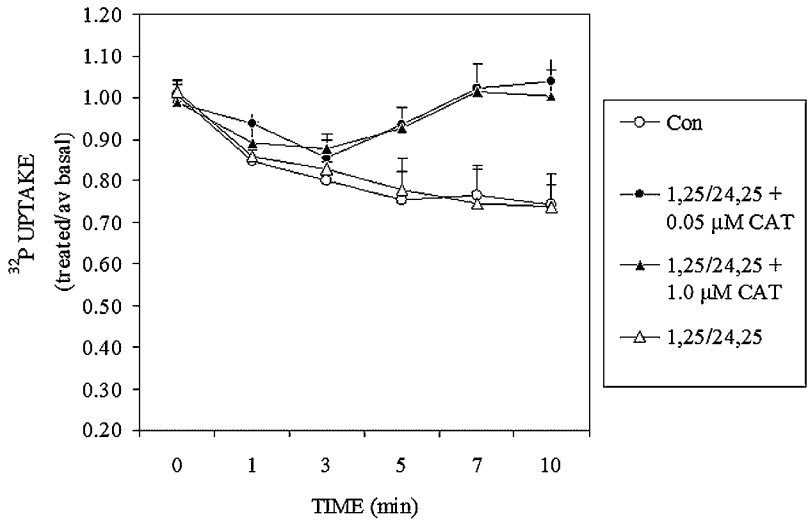

Fig. 2. Time course study of ^{32}P uptake and effect of exogenous catalase in isolated intestinal cells. Enterocytes were isolated and exposed to radionuclide as described in the legend of Fig. 1. Aliquots of cell suspensions were pipetted into four fresh tubes, two of which contained 0.05 or 1.0 μM bovine catalase. Samples were removed at T = −5 and −1 min to establish basal uptake. At T = 0 min, the cells were treated with vehicle or steroids to produce the following incubation conditions: (1) controls plus catalase (○–○; n = 5); (2) 300 pM $1,25(OH)_2D_3$ plus 6.5 nM $24,25(OH)_2D_3$ plus 0.05 μM catalase (●–●; n = 5); (3) 300 pM $1,25(OH)_2D_3$ plus 6.5 nM $24,25(OH)_2D_3$ plus 1.0 μM catalase (▲–▲; n = 5); (4) 300 pM $1,25(OH)_2D_3$ plus 6.5 nM $24,25(OH)_2D_3$ (△–△ n = 5). At T = 1, 3, 5, 7, and 10 min, 100 μl samples were taken and pipetted into 900 μl of ice-cold GBSS. Cell samples were centrifuged (1,000*g*, 5 min), supernatants decanted, and pellets analyzed for radioactivity activity and protein. Radionuclide was related to corresponding cellular protein in each aliquot, and then normalized to average basal cpm/μg protein. Data are presented as mean treated/average basal ratios ± SEM.

of binding $24,25(OH)_2D_3$ rather than removal of H_2O_2.

^{32}P Uptake and Effect of Catalase Inhibitor

Figure 3 illustrates the results of experiments designed to test whether addition of a triazole catalase inhibitor duplicates the inhibitory effect of $24,25(OH)_2D_3$. Vehicle controls showed a general decrease in ^{32}P levels with time (open circles), as noted above. Incubation of cells in the presence of both $1,25(OH)_2D_3$ and catalase inhibitor, as well as $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$ resulted in ^{32}P uptake levels that were not significantly different than controls (Fig. 2). Addition of $1,25(OH)_2D_3$ alone, resulted in enhanced levels of ^{32}P uptake that were evident within 1 min of hormone addition, and which became statistically significant at 3 to 10 min ($P = 0.01$ to 0.001), relative to corresponding controls or corresponding samples from cells treated with both steroids. Catalase inhibitor mimicked the $24,25(OH)_2D_3$-mediated inhibition of $1,25(OH)_2D_3$, in that ^{32}P uptake was equivalent to levels observed in controls (Fig. 3).

^{32}P Uptake and 4α-Phorbol 12-Myristate 13-Acetate

We have previously reported that the $1,25D_3$-MARRS receptor mediates enhanced phosphate uptake through the PKC signaling pathway (Zhao and Nemere, 2002) and can be inhibited by calphostin C [Sterling and Nemere, 2005]. To test whether the production of H_2O_2 mediated by $24,25(OH)_2D_3$ can act to inhibit PKC signaling independently of the demonstrated oxidation of the $1,25D_3$-MARRS receptor [Nemere et al., 2006], 4β-Phorbol 12-myristate 13-acetate (PMA) was used to activate PKC. Vehicle controls plus H_2O_2 showed a general decrease in ^{32}P levels with time (open circles), but the decline was equivalent to that seen in the absence of oxidant. Addition of PMA alone (positive control), resulted in enhanced levels of ^{32}P uptake that were evident within 1 min after addition, and which became statistically significant at 5 to 10 min ($P = 0.02$ to 0.001), relative to corresponding controls. Incubation of cells in the presence of both hydrogen peroxide and PMA, resulted in ^{32}P uptake levels that were not significantly different than controls (Fig. 4).

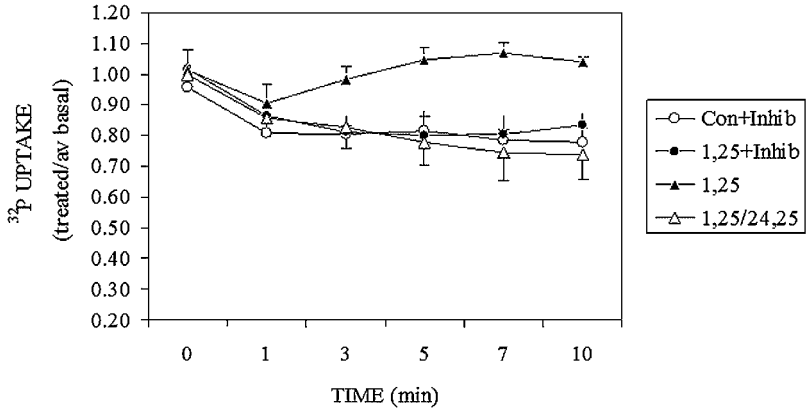

Fig. 3. Time course study of ^{32}P uptake and effect of catalase inhibitor in isolated intestinal cells. Enterocytes were isolated and exposed to radionuclide as described in the legend to Figure 2. Aliquots of cell suspension were pipetted into four fresh tubes, two of which contained 1 μM catalase inhibitor (3-amino-1,2,4-triazole). Samples were removed at T = −5 and −1 min to establish basal uptake. At T = 0 min, the cells were treated with vehicle or steroids to produce the following incubation conditions: (1) controls plus catalase inhibitor (○–○; n = 5); (2) 300 pM $1,25(OH)_2D_3$ plus catalase inhibitor (●–●; n = 5); (3) 300 pM $1,25(OH)_2D_3$ (▲–▲; n = 5); (4) 300 pM $1,25(OH)_2D_3$ plus 6.5 nM $24,25(OH)_2D_3$ (△–△; n = 5). At T = 1, 3, 5, 7, and 10 min, 100 μl samples were taken and pipetted into 900 μl of ice-cold GBSS. Cell samples were centrifuged (1,000*g*, 5 min), supernatants decanted, and pellets analyzed for radioactivity activity and protein. Radionuclide was related to corresponding cellular protein in each aliquot, and then normalized to average basal cpm/μg protein. Data are presented as mean treated/average basal ratios ± SEM.

Effect of PMA on PKC Activity in the Absence and Presence of H_2O_2

Earlier studies [Nemere et al., 2006] indicated that inactivation of the $1,25D_3$-MARRS receptor by either $24,25(OH)_2D_3$ or 50 μM H_2O_2 required 5–20 min of incubation, while inhibition of phosphate uptake occurred within 1 min [Zhao and Nemere, 2002; and Figures 1–3 in the current study]. As shown in Figure 4, direct stimulation of phosphate uptake with phorbol ester was rapidly inhibited by H_2O_2, suggesting

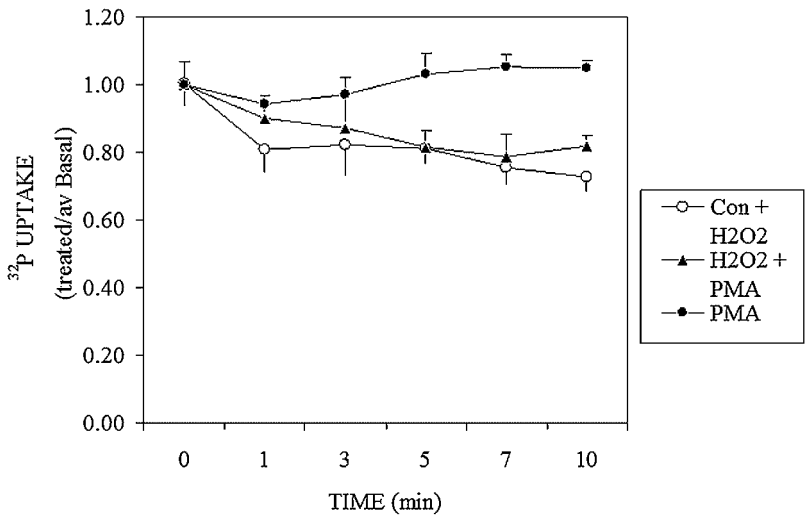

Fig. 4. Time course study of ^{32}P uptake and effect of 4β-Phorbol 12-myristate 13-acetate (PMA) in isolated intestinal cells. Enterocytes were isolated and exposed to radionuclide as described in the legend to Figure 2. Aliquots of cell suspension were pipetted into three fresh tubes, two of which contained 50 μM H_2O_2. Samples were removed at T = −5 and −1 min to establish basal uptake. At T = 0 min, the cells were treated with 100 nM PMA or vehicle to produce the following incubation conditions: (1) controls plus 50 μM H_2O_2, (○–○; n = 3); (2) 100 nM PMA plus 50 μM H_2O_2 (●–●; n = 3); (3) 100 nM PMA (▲–▲; n = 3) At T = 1, 3, 5, 7, and 10 min, 100 μl samples were taken and pipetted into 900 μl of ice-cold GBSS. Cell samples were centrifuged (1,000*g*, 5 min), supernatants decanted, and pellets analyzed for radioactivity activity and protein. Radionuclide was related to corresponding cellular protein in each aliquot, and then normalized to average basal cpm/μg protein. Data are presented as mean treated/average basal ratios ± SEM.

that the oxidant might directly inhibit PKC activation. Experiments were therefore performed to assess this possibility.

Figure 5 illustrates the time course of PKC activation in response to various treatments of intestinal epithelial cells. Vehicle controls incubated in the presence of hydrogen peroxide essentially had PKC levels that did not change with time (open circles), while addition of $1,25(OH)_2D_3$ increased enzyme activity to 120% of controls after 5 min of incubation. In cells treated with PMA, PKC levels were noticeably greater within 1 min, and became significantly greater than corresponding controls at 3 to 5 min ($P < 0.05$; Fig. 5). At 5 min of incubation, PMA-treated cells showed PKC levels 148% of corresponding controls. Incubation of cells in the presence of both PMA and H_2O_2 resulted in PKC levels that were not significantly different than controls (Fig. 5).

DISCUSSION

The present study provides additional evidence that reactive oxygen species generated through the binding of $24,25(OH)_2D_3$ to catalase mediate the inhibition of stimulated phosphate uptake. Preincubation of isolated intestinal epithelial cells with Ab 365—previously shown to recognize catalase [Larsson et al., 2006]—prevented inhibition of stimulated phosphate uptake. This is most likely due to blocking binding of $24,25(OH)_2D_3$ to its cell-surface recognition moiety, as the antibody itself does not alter catalase activity. Likewise, commercially available catalase was found to lose enzyme activity rapidly, so that addition to enterocytes most likely presented a situation where excess, soluble "receptor" effectively competed for binding to $24,25(OH)_2D_3$.

It is also particularly noteworthy that inhibition of catalase activity by a completely unrelated compound mimicked the action of $24,25(OH)_2D_3$. In an earlier work [Nemere et al., 2006], we demonstrated that oxidation of thiols on the $1,25D_3$-MARRS receptor accounted for a portion of the inhibition initiated by $24,25(OH)_2D_3$ binding to catalase, and that this was closely paralleled by the observed effects of exogenous H_2O_2. However, this pathway of feed back became evident after 5 min of exposure of cells to either $24,25(OH)_2D_3$ or H_2O_2, while blockade of phosphate uptake

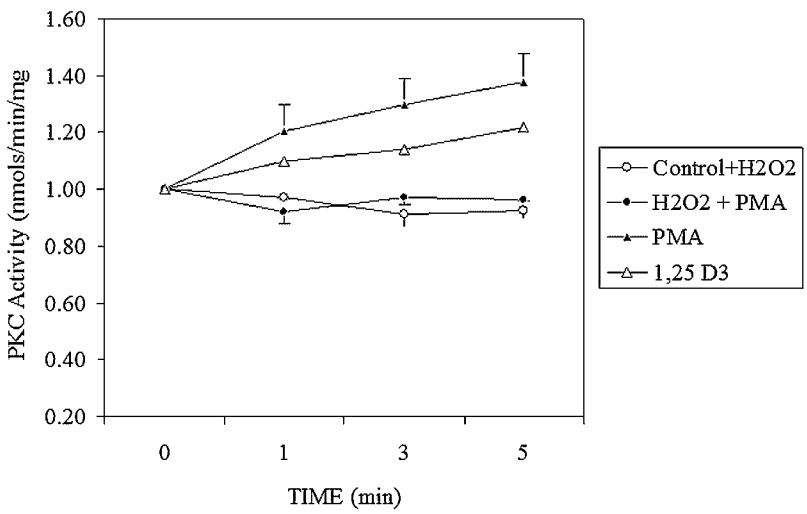

Fig. 5. Time course study of PKC activation by 4β-Phorbol 12-myristate 13-acetate (PMA) in isolated intestinal cells. At T = 0 min, the cells were treated with H_2O_2, PMA, or $1,25(OH)_2D_3$ to produce the following incubation conditions: (1) Control plus 50 μM H_2O_2 (○–○; n = 3); (2) 50 μM H_2O_2 plus 100 nM PMA (●–●; n = 3); (3) 100 nM PMA (▲–▲; n = 3); (4) 300 pM $1,25(OH)_2D_3$ (△–△; n = 2). Cell suspensions were sampled at T = 0, 1, 3, and 5 min. After incubation, as described above, the cells were centrifuged at 1,000*g* at 4°C for 10 min, the supernatant was decanted, and the pellet was stored at −20°C until analysis could be performed. Samples were placed on ice, 500 μl of chilled double-distilled water was added, and the samples homogenized. Samples were transferred into microfuge tubes on ice. PKC activity was analyzed using [γ ^{32}P] ATP, and the substrate peptide [QKRPSQRSKYL]. Aliquots of the incubation mixtures were spotted onto phosphocellulose disks, washed and placed into scintillation vials for determination of radioactivity.

by $24,25(OH)_2D_3$ was seen within 1 min of incubation [Zhao and Nemere, 2002]. We therefore considered the possibility that PKC, the signal transduction mediator of $1,25(OH)_2D_3$-stimulated phosphate uptake might also be affected by reactive oxygen species. To avoid involving the $1,25D_3$-MARRS receptor pathway, we treated isolated intestinal cells with phorbol ester, a direct stimulator of PKC activity. As anticipated, phorbol ester stimulated phosphate uptake, and pre-incubation of cells with H_2O_2 abolished enhanced ion uptake. The inhibition occurred within 1 min, a time consistent with inhibition by $24,25(OH)_2D_3$. These observations are also in keeping with the earlier report that $24,25(OH)_2D_3$ inhibits $1,25(OH)_2D_3$-induced increases in PKC activity [Nemere et al., 2000]. Other labs have also reported distinct effects of $24,25(OH)_2D_3$ on osteosarcoma cells [Yukihiro et al., 1994; Takeuchi and Guggino, 1996], mouse osteoblasts [Grosse et al., 1993], and rat chondrocytes (Pedrozo et al., 1999]. Our work has relevance at the physiological level: in an agricultural context, our findings have applicability to raising poultry in that the phosphate content of the manure can upset the ecological balance in waterways. Thus, dietary antioxidants may be one approach to the problem. Perhaps of greater interest are the biomedical aspects, since dietary antioxidants have been correlated with bone health [Lean et al., 2003]. In addition, $24,25(OH)_2D_3$ has been demonstrated to have hypocalcemic effects in vivo [Maeda et al., 1987; Tryfonidou et al., 2002], to play a central role in bone formation [Somjen et al., 1983; Ono et al., 1996] and healing [Fine et al., 1985], and to effectively mitigate hyperparathyroidism [Carpenter et al., 1996]. The evolutionary importance of $24,25(OH)_2D_3$ effects are underscored by studies in marine cod, where it has been found that the metabolite actively decreases intestinal calcium transport in marine fish [Sundell and Bjornsson, 1990; Larsson et al., 1995], thereby offering protection from a hypercalcemic environment. The combined evidence supports the pivotal role of redox signaling in the vitamin D endocrine system.

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