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Catalase Activity Mediates the Inhibitory Actions of 24,25 Dihydroxyvitamin D_3

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CATALASE ACTIVITY MEDIATES THE INHIBITORY ACTIONS OF 24,25 DIHYDROXYVITAMIN D_3

by

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ABSTRACT

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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 the $24,25(OH)_2D_3$ 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 a putative $24,25(OH)_2D_3$ binding protein was found to neutralize the inhibitory effect of the steroid on $1,25(OH)_2D_3$-mediated ^{32}P uptake ($P<0.05$ to 0.001 at T=3-10 min of incubation). Incubation of cells in the presence of 50 nM catalase was also found to alleviate inhibition at T=5-10 min of incubation. In another series of experiments, isolated intestinal epithelial cells were incubated as controls or with $1,25(OH)_2D_3$, each in

Cells exposed to hormone alone again showed an increased accumulation of ^{32}P from T=5-10 min, 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 100 nM phorbol-13-myristate (PMA) increased ^{32}P uptake to 143% of controls, while cells pretreated with 50 μM H_2O_2 prior to PMA did not exhibit increased uptake. Likewise, PMA significantly increased PKC activity at T=1-3 min ($P< 0.05$, relative to corresponding controls), 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.

(48 pages)

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Sven L. Peery

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CHAPTER I

LITERATURE REVIEW

Background

When vitamin D was discovered in 1922 it was considered a nutritional factor. It has since been categorized as a pre-hormone rather than a vitamin. A major reason for this belief is that most of the vitamin D utilized by higher animals is manufactured in the skin by a photochemical process. Under these conditions, vitamin D is not required in the diet, so it does not meet the definition of a vitamin. Moreover, it is further metabolized into more active forms, which is a property of a pre-hormone. Vitamin D is the name applied to two fat-soluble substances that are also known as cholecalciferol (in animals) and ergocalciferol (in plants and yeasts) in its inactive state. For activation, cholecalciferol must first be converted into $25(OH)D_3$ in the liver where it is hydroxylated at carbon-25 (see appendix A for a complete list of abbreviations.). In the kidney, $25(OH)D_3$ is either hydroxylated at carbon-1 or carbon-24. $1,25(OH)_2D_3$ is a hormonally active secosteroid (a compound in which the B ring has been cleaved). $24,25(OH)_2D_3$ is another metabolite which was thought to be hormonally inactive, but is beginning to be appreciated as a hormone as well [Henry and Norman, 1978; Ono et al., 1996; Somjen et al., 1983].

The liver appears to be the major site of 25-hydroxylation in mammals. In the chicken however, the 25-hydroxylase enzymes exist in extra-hepatic sites such as the kidney and the intestines. It is generally thought by many that although this steroid ($25(OH)D_3$) is a major circulating metabolite of cholecalciferol it must undergo chemical modification to $1,25(OH)_2D_3$ or $24,25(OH)_2D_3$ before it can function as a hormone.

However, reports by Olson and DeLuca [1969] and Phadnis and Nemere [2003] suggest that $25(OH)_2D_3$ may have activity in promoting calcium transport (see below).

A well-studied vitamin D metabolite target organ is the intestine. In the intestine, the nuclear receptor for the metabolite, $1,25(OH)_2D_3$ may regulate the biosynthesis of vitamin D dependent calcium binding protein (CaBP), termed calbindin-D_{28k} which binds Ca^{2+} with high affinity [Minghetti et al., 1988; Corradino et al., 1968; Corradino and Wasserman, 1968]. However, it may be that the increase in calcium absorption stimulates the CaBP synthesis, since Spencer et al. [1976] found that enhanced transport preceded the appearance of CaBP.

Mechanisms of Biological Action of Vitamin D Metabolites

Thus far, the three known active metabolites of Vitamin D are $25(OH)D_3$; $1,25(OH)_2D_3$; $24,25(OH)_2D_3$ (see table 1 below for summary of metabolites). While $25(OH)D_3$ causes an increase in calcium uptake in rat duodena [Olson and DeLuca 1969], it has also been shown to increase calcium transport in the perfused duodenal loops of chicks. In isolated chick enterocytes and perfused duodenal loops, Zhao and Nemere [2002] reported that $1,25(OH)2D_3$ increases phosphate uptake and transport. On the other hand, $24,25(OH)_2D_3$ has an inhibitory effect when administered in the presence of $1,25(OH)_2D_3$ [Nemere 1996a; Nemere et al., 2002; Zhao and Nemere, 2002]. The most commonly studied metabolite of vitamin D is $1,25(OH)_2D_3$. It has been shown that this metabolite acts both by genomic and membrane-initiated actions [Nemere, 1996b].

Table 1. Vitamin D metabolite action summary

Metabolite	Sight of activation	Proposed Action	Reference
$25(OH)D_3$	Liver	↑ CA^{2+} uptake in rat & chick duodena	Olson EB, DeLuca HF 25-hydroxycholecalciferol: direct effect on calcium transport. Science. 1969 Jul 25;165(891):405-7.
$1,25(OH)_2D_3$	Kidney	↑ phosphate uptake and transport in intestine	Zhao B. and Nemere I. $1,25(OH)_2D_3$-mediated phosphate uptake in isolated chick intestinal cells: effect of $24,25(OH)_2D_3$, signal transduction activators, and age. J. Cell Biochem. 2002;86(3):497-508
$24,25(OH)_2D_3$	Kidney	↓ phosphate uptake in the presence of $1,25(OH)_2D_3$ in intestine	Nemere, I. 1996a. Apparent non-nuclear regulation of intestinal phosphate transport: effects of 1,25-dihydroxyvitamin D_3, 24,25-dihydroxyvitamin D_3, and 25-hydroxyvitamin D_3.Endocrinology. 1996 Jun;137(6):2254-61.

Various genomic (nuclear) actions of vitamin D have been found to promote synthesis of calcium transport pathway elements such as ion carriers. Early evidence for lysosomes as vesicular carriers came from electron microscopic studies. Jande and Brewer [1974] observed that vitamin D repletion of vitamin D deficient chicks resulted in the proliferation of intestinal epithelial cell lysosomes. Warner and Coleman [1975] used X-ray probe analysis in conjunction with electron microscopy, and found that during transport, calcium was found in discrete localizations suggesting a vesicular accumulation, as opposed to a diffuse cytoplasmic route.

Nemere et al. [1986] using biochemical techniques to study subcellular organelles reported that regardless of the vitamin D status, the highest levels of ^{45}Ca were found in lysosomal fractions during transport. Lysosomal fractions were also the only subcellular

compartment to exhibit a $1,25(OH)_2D_3$-induced increase in ^{45}Ca content. Time course [Nemere and Norman, 1988] and dose response studies [Nemere, 1999] further supported the vesicular carrier hypothesis.

In two separate studies Nemere et al. [1986c, 1991] reported that lysosomes were found to be enriched in calbindin-D_{28k}. Using ELISA in combination with subcellular fractionation studies, lysosomal fractions were found to be enriched in calbindin-D_{28k} although most of the protein remained soluble. It was recognized that soluble CaBP could be due to a homogenization effect. Using electron microscopy Nemere et al. [1991] found very little cytoplasmic calbindin D_{28k} while most of the immuno-reactive protein was in small transport vesicles and lysosomes.

Microtubules are also deemed to be a potential regulatory point for the genomic actions of vitamin D. An estimation of the α-tubulin mRNA by dot blot analysis [Nemere et al., 1987] revealed that in chick intestine after $1,25(OH)_2D_3$ treatment there were decreasing levels of message but increasing levels of protein. The inverse relation between α-tubulin and its message has been ascribed to auto regulation [Cleveland and Sullivan, 1985].

Membrane Initiated Actions

It can be argued that when an animal reaches a Ca^{2+} deficient state, it would be beneficial to obtain crucial Ca^{2+} rapidly from the diet without delay due to gene transcription in the nucleus. Given the pivotal role of Ca^{2+} in muscle contraction, nerve impulse conduction, and other physiologic phenomena, such a rapid response could be life saving for the organism.

The first evidence for rapid effects was obtained 30 years ago [Toffolon et al., 1975]. Toffolon et al. [1975] reported that within 30 min of $1,25(OH)_2D_3$ administration in vivo, subsequently prepared everted gut sacs from rats responded with enhanced calcium transport, relative to control preparations. The authors speculated that the secosteroid hormone might become inserted into the lipid bilayer of the apical membrane of the intestinal cells and thereby exert a direct membrane effect.

A direct rapid effect of $1,25(OH)_2D_3$ on calcium uptake was first observed in isolated intestinal epithelial cells from the rat, where the authors postulated that a membrane-localized receptor might be responsible for initiating the effects observed [Nemere and Szego, 198l a,b]. Using rat enterocytes Lieberherr et al. [1989] reached a similar conclusion regarding the need for a cell surface receptor to mediate developmental changes in $1,25(OH)_2D_3$ stimulated phosphoinositide metabolism (apparent within 5 sec of hormone treatment). Further downstream of the initial phosphoinositide turnover stimulated by $1,25(OH)_2D_3$, protein kinase C (PKC) has also been found to be involved in mediating rapid responses in the rat colonic epithelium and Caco- 2 cells [Wali et al., 1990, 1992; Khare et al., 1994].

A similar phenomenon has been found in fresh water fish that live in a relatively low Ca^{2+} environment: $1,25(OH)_2D_3$ induces rapid Ca^{2+} transport in perfused intestinal preparations while no such effect is observed in marine fish that live in a high Ca^{2+} environment [Sundell and Bjornsson, 1990]. Physiologically relevant concentrations of $24,25(OH)_2D_3$ however, decreased Ca^{2+} transport in the perfused intestine of the Atlantic cod, a marine fish [Sundell and Bjornsson, 1990; Larsson et al., 1995].

The perfused chick duodenal loop is a valuable system for the study of rapid actions of $1,25(OH)_2D_3$ and $24,25(OH)_2D_3$, including modulation of intestinal Ca^{2+} and phosphate transport [Nemere et al., 1984, 1986, 1996a]. In this system it was observed that $1,25(OH)_2D_3$ acts at the basal lateral membrane (BLM) surface and not at the brush border [Nemere et al., 1984; Nemere 1996a,b]. This observation suggests binding to a membrane receptor, rather than non-specific membrane insertion by $1,25(OH)_2D_3$ as the initiating effect. The biphasic dose response curve for both steroid mediated Ca^{2+} and phosphate transport is also characteristic of membrane receptors.

Evidence for the Biological Effect of $24,25(OH)_2D_3$

The metabolite $24,25(OH)_2D_3$ is preferentially produced when an animal is calcium-, phosphate-, and $1,25(OH)_2D_3$ replete. Thus, it may be an endogenous inhibitor of $1,25(OH)_2D_3$. In this regard, it has been shown that $24,25(OH)_2D_3$ suppresses the rapid actions of $1,25(OH)_2D_3$ on phosphate and calcium transport in the chick intestine [Nemere, 1996a, 1999]. At a cellular level $24,25(OH)_2D_3$ has been found to inhibit the rapid membrane initiated effects of $1,25(OH)_2D_3$ on opening of calcium channels in osteoblasts and osteosarcoma cells [Yukihiro et al., 1994; Khoury et al., 1995; Takeuchi and Guggino, 1996] and PKC activation in chick enterocytes [Nemere, 1999].

Proposed Mechanism of $24,25(OH)_2D_3$ Action

Growing evidence has shown that $24,25(OH)_2D_3$ has specific, physiologically significant actions that are distinct from $1,25(OH)_2D_3$. Larsson et al. [2006] recently

identified catalase as an endogenous $24,25(OH)_2D_3$ binding protein. It should be noted that catalase has been found tightly bound to the cell surface in other tissues [Watanabe et al., 2003]. This finding suggests a mechanism for signal transduction, acting as a membrane recognition moiety that subsequently directs the steroid to intracellular organelles. The peroxisomal marker enzyme catalase, is well known for its ability to eliminate hydrogen peroxide. Hydrogen peroxide in turn, has recently been implicated as a newly found signal transducer [Wantanabe et al., 2003; Waypa et al., 2002; Wood et al., 2003; Yano and Yano, 2002]. Nemere et al. [manuscript submitted] went on to investigate the potential role of reactive oxygen species in $24,25(OH)_2D_3$ signaling. This study found that the functional consequence of $24,25(OH)_2D_3$ binding was to decrease catalase activity, with an ensuing increase in hydrogen peroxide production, which in turn inhibited $1,25(OH)_2D_3$-stimulated phosphorus uptake. These findings suggested that a target of inhibitory oxidation might be the $1,25D_3$-MARRS protein, which contains two thioredoxin folds [Nemere et al., 2004]. The reduced thiols are most likely responsible for protein-protein interactions that allow for positively cooperative binding [Larsson and Nemere, 2003], and may facilitate movement of the $1,25D_3$-MARRS protein to the nucleus following ligand binding [Nemere et al., 2000; Rohe et al., 2005]. Incubation of enterocytes with either $24,25(OH)_2D_3$ or hydrogen peroxide for greater than 5 minutes, reduced binding of $1,25(OH)_2D_3$ by the $1,25D_3$-MARRS receptor. Binding could however be restored in the presence of dithiothreitol indicating the involvement of thiol groups. Binding by the VDR in the same cells was not decreased by $24,25(OH)_2D_3$ but actually increased after addition of hydrogen peroxide. The authors concluded with evidence that enhanced binding by the VDR after exposure to reactive oxygen species

suggested the existence of a mechanism to preserve transcriptional effects mediated by the VDR [Nemere et al., 2006 manuscript submitted].

Thesis Proposal

With mounting evidence suggesting nongenomic actions of $24,25(OH)_2D_3$, I tested the hypothesis that pro-oxidant states inhibit $1,25(OH)_2D_3$ action on uptake of phosphorus, and that anti-oxidant conditions promote $1,25(OH)_2D_3$-mediated uptake of phosphorus. The specific aims were as follows:

1. To determine the effect of Ab 365 (against the $24,25(OH)_2D_3$ binding protein) on ^{32}P uptake in intestinal cell suspensions. This represented an anti-oxidant condition.
2. To determine the effect of exogenous catalase on ^{32}P uptake of cells treated with hormone. This represented an additional anti-oxidant condition:
3. To determine the effect of a catalase inhibitor on ^{32}P uptake in intestinal cell suspensions (A pro-oxidant condition).
4. To determine whether pro-oxidant conditions inhibited PKC signaling directly, rather than by way of inhibiting ligand binding to the $1,25D_3$-MARRS receptor. Phorbol ester (a direct activator of PKC) stimulates phosphate uptake without prior activations of the $1,25D_3$-MARRS receptor.

A schematic diagram of the functional cellular relationship between the $1,25D_3$-MARRS receptor and catalase is presented in Figure 1.

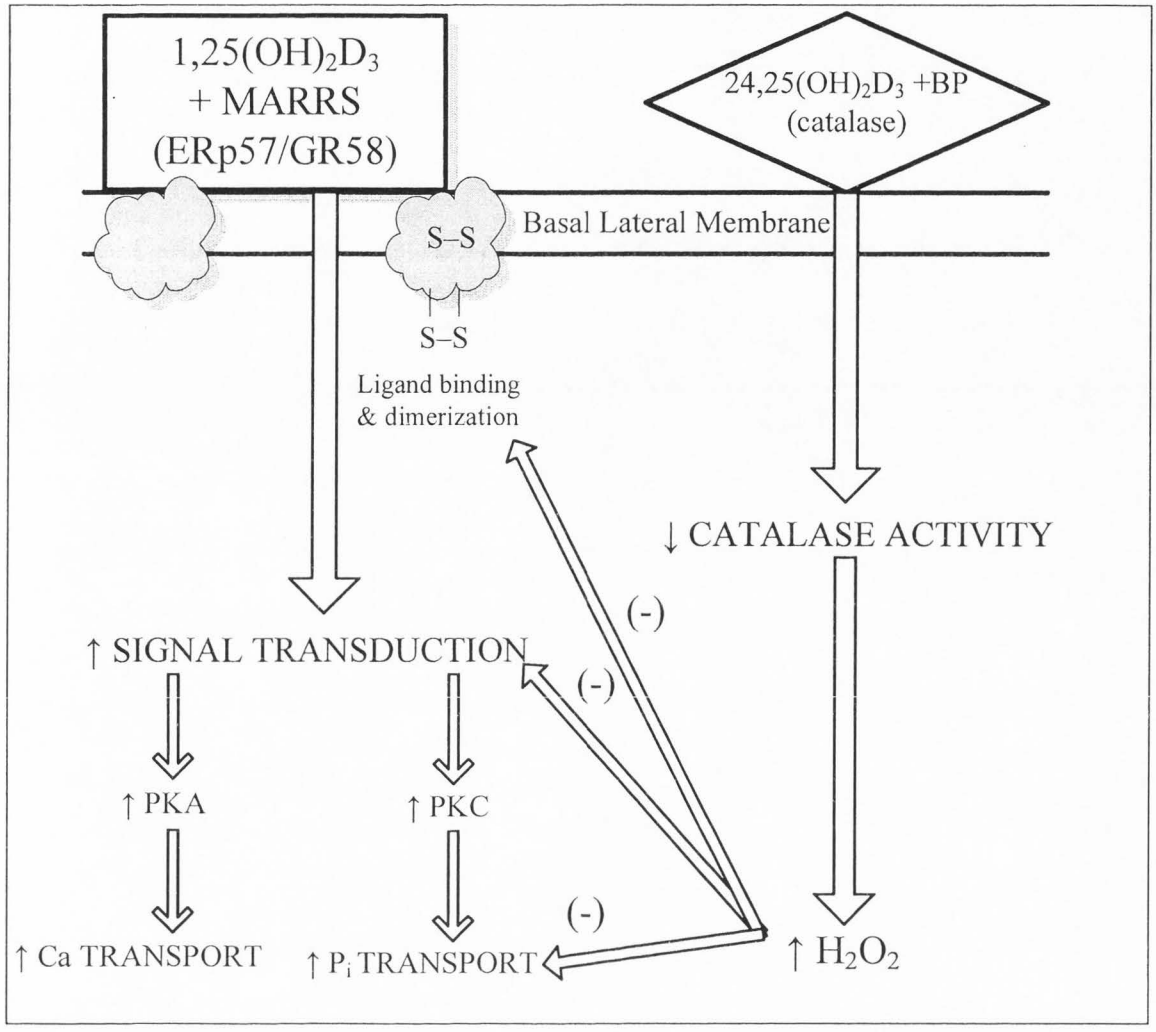

Fig. 1. Model of endocrine feedback of $24,25(OH)_2D_3$ on the rapid actions of $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ binds to the $1,25D_3$-MARRS receptor in the basal lateral membrane. The two thioredoxin homology domains provide a means for protein-protein interactions, including positive coopertivity in ligand binding [Larsen & Nemere 2003a,b]. Binding of $1,25(OH)_2D_3$ stimulates at least two signal transduction pathways. Increased PKA activity stimulates Ca uptake and transport, [Nemere 2002; Phadnis & Nemere 2003] while increased PKC activity stimulates P_i uptake and transport [Zhao & Nemere; Nemere et al 2004]. The metabolite $24,25(OH)_2D_3$ is known to inhibit $1,25(OH)_2D_3$ stimulated uptake and transport of these ions [Nemere 1999]. By binding to catalase, $24,25(OH)_2D_3$ decreases enzyme activity and increases H_2O_2 production [Nemere et al. manuscript submitted], thereby oxidizing sulfhydryls in the $1,25D_3$–MARRS receptor to inhibit $1,25(OH)_2D_3$ binding. In addition, PKC signal transduction is also directly inhibited.

CHAPTER II

MATERIALS AND METHODS

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/l00g 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 (Nemere and Campbell, 2000). The duodenal loop was transferred to 30 ml of Solution A [medium first described by Weiser [1973], 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 intestinal segment was then transferred to fresh buffer and the process was repeated twice with 30 ml aliquots of Solution A. The cells were collected from the pooled isolation media by centrifugation at 500 x 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 $CaCl_2$ pH 7.0, 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)$_2$D$_3$ Binding Protein

Fourteen milliliters of the cell suspension in GBSS was removed to a polypropylene tube (Falcon, Fischer Scientific) 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, three of which contained Ab 365 (1/100 dilution, final concentration) against the 24,25$(OH)_2D_3$ binding protein. One hundred-microliter aliquots 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$(OH)_2D_3$ plus Ab 365; (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 1000 x 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 in duplicate 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 treatment conditions were (1) vehicle controls plus catalase; a.(2) 300 pM $1,25(OH)_2D_3$ plus 6.5 nM $24,25(OH)_2D_3$ plus 50 nM catalase; or b (3) 300 pM $1,25(OH)_2D_3$ plus 6.5 nM $24,25(OH)_2D_3$ plus 1 µM catalase; (4) 300 pM $1,25(OH)_2D_3$ plus 6.5 nM $24,25(OH)_2D_3$.

^{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 (Sigma). At T= 0, suspensions were treated with vehicle or steroid. The treatment groups were (1) controls plus catalase inhibitor; (2) 300 pM $1,25(OH)_3D_3$; and (3) 300 pM $1,25(OH)_3D_3$ plus catalase inhibitor.

^{32}P Uptake and 4β-Phorbol 12-myristate 13-acetate

Cell suspensions were combined with $H_3{}^{32}PO_4$ as described above and aliquots pipetted into fresh tubes, two of two of which contained 50 μM H_2O_2. At T= 0 min, cells were exposed to either the vehicle ethanol or 100 nM 4β-Phorbol 12-myristate 13-acetate (PMA; Sigma). The treatment conditions were (1) vehicle controls plus hydrogen peroxide; (2) hydrogen peroxide plus PMA; (3) PMA.

Calculations

For each time course, basal counts per minute per μg of protein were averaged, and used to normalize specific ^{32}P uptake during the corresponding treated phase.

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 (PMA; Sigma), or PMA 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 1000 x 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 μl 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. Protein concentrations were determined using the Bradford reagent as discussed above.

Statistical Analyses

Statistical comparisons between treatment groups were made by Sigma plot 9.0. using a two way ANOVA. Statistical significance was set at $P<0.05$. Results are expressed as Mean ± SEM for 3-8 independent experiments. See appendix B for a detailed summary of statistical measures.

CHAPTER III

RESULTS

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

Figure 2 shows the results of experiments designed to test whether neutralization of the $24,25(OH)_2D_3$ binding protein affects inhibition of the $1,25(OH)_2D_3$ response by the 24-hydroxylated steroid. 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]. 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, and which became statistically significant at 3 to10 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. 2), also as previously reported [Zhao and Nemere, 2002]. However, incubation of cells in the presence of Ab 365 and both steroids reversed the inhibition (Fig. 2). Radionuclide levels were noticeable greater in such cell suspensions within 1 min, and became significantly greater than corresponding incubations without antibody at 3 to 10 min ($P<0.001$) (Fig. 3). 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.

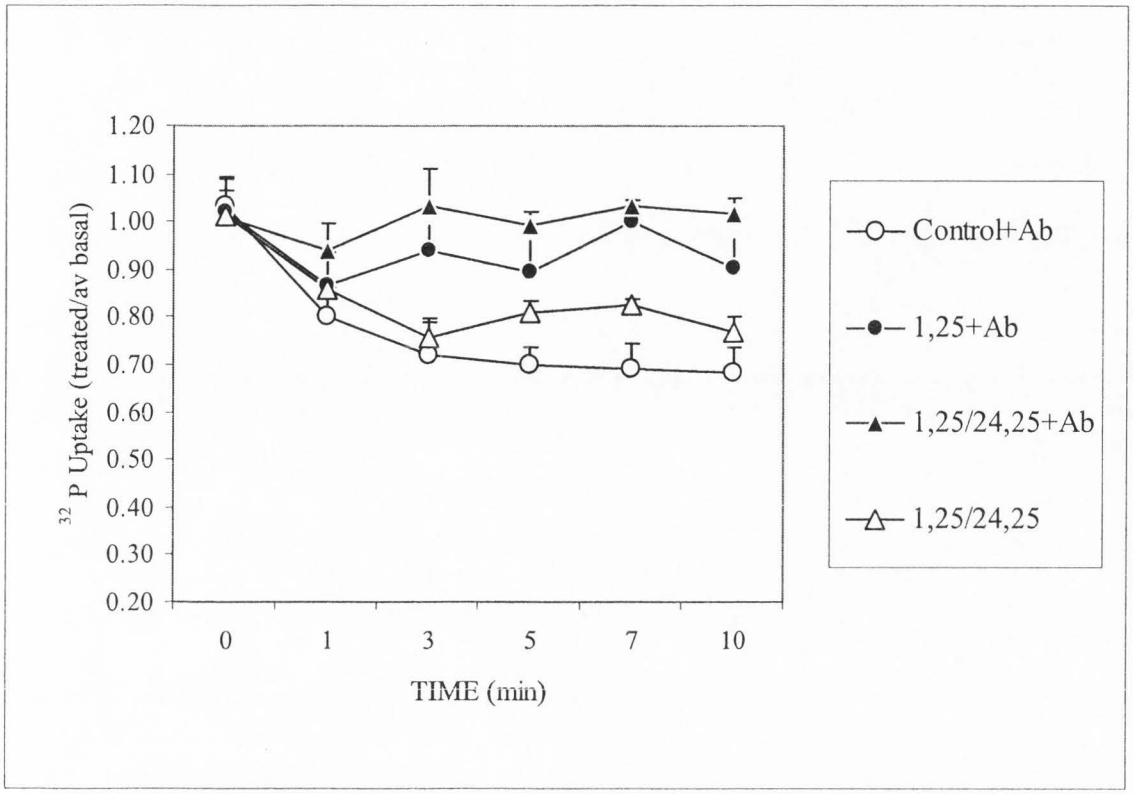

Fig. 2. Time course of ^{32}P Uptake and effect of Ab 365 against the $24,25(OH)_2D_3$ binding protein. Enterocytes isolated by citrate chelation were resuspended in Gey's Balanced Salt Solution (GBSS; 23° C), and combined with 2 μCi/ml of $H_3{}^{32}PO_4$, at which point the time course was initiated. 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 $24,25(OH)_2D_3$ (Δ—Δ; n=7); (4) 300 pM $1,25(OH)_2D_3$ plus 6.5 nM $24,25(OH)_2D_3$ plus antibody 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 (1000 x 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.

^{32}P uptake and Effect of Exogenous Catalase

Figure 3 illustrates the results of experiments designed to test whether addition of exogenous catalase overcomes 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. uptake levels that were not significantly different than controls (Fig. 3), also as previously reported [Zhao and Nemere, 2002]. Exogenous catalase reversed the $24,25(OH)_2D_3$-mediated inhibition of $1,25(OH)_2 D_3$. 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 to10 min ($P = 0.02$-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.

^{32}P uptake and Effect of Catalase Inhibitor

Figure 4 depicts the results of experiments designed to test whether addition of 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. 4). Catalase inhibitor mimicked the $24,25(OH)_2D_3$-mediated

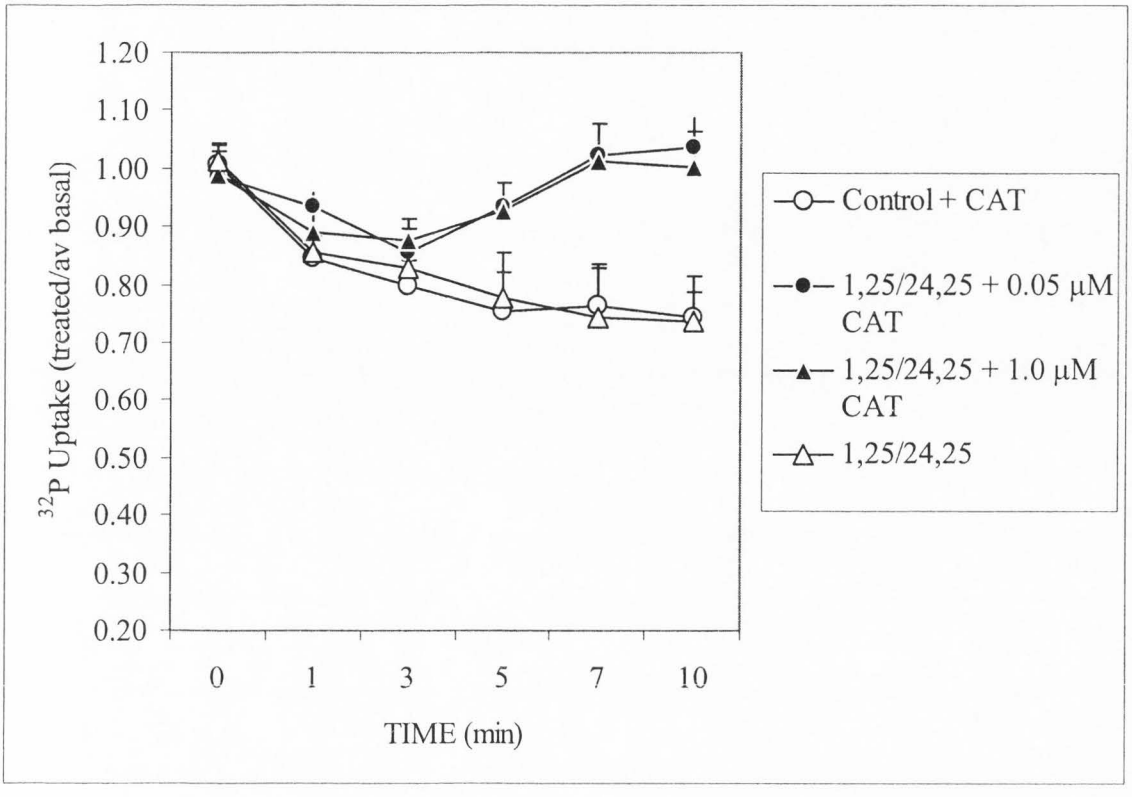

Fig. 3. Time course study of ^{32}P uptake and effect of exogenous catalase in isolated intestinal cells. Enterocytes were isolated and exposed to radionuclide as described in the legend to Fig. 2. Aliquots of cell suspensions were pipetted into four fresh tubes, two of which contained 0.05 or 1.0 μM 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 $24,25(OH)_2D_3$ (Δ—Δ; 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 (1000 x 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.

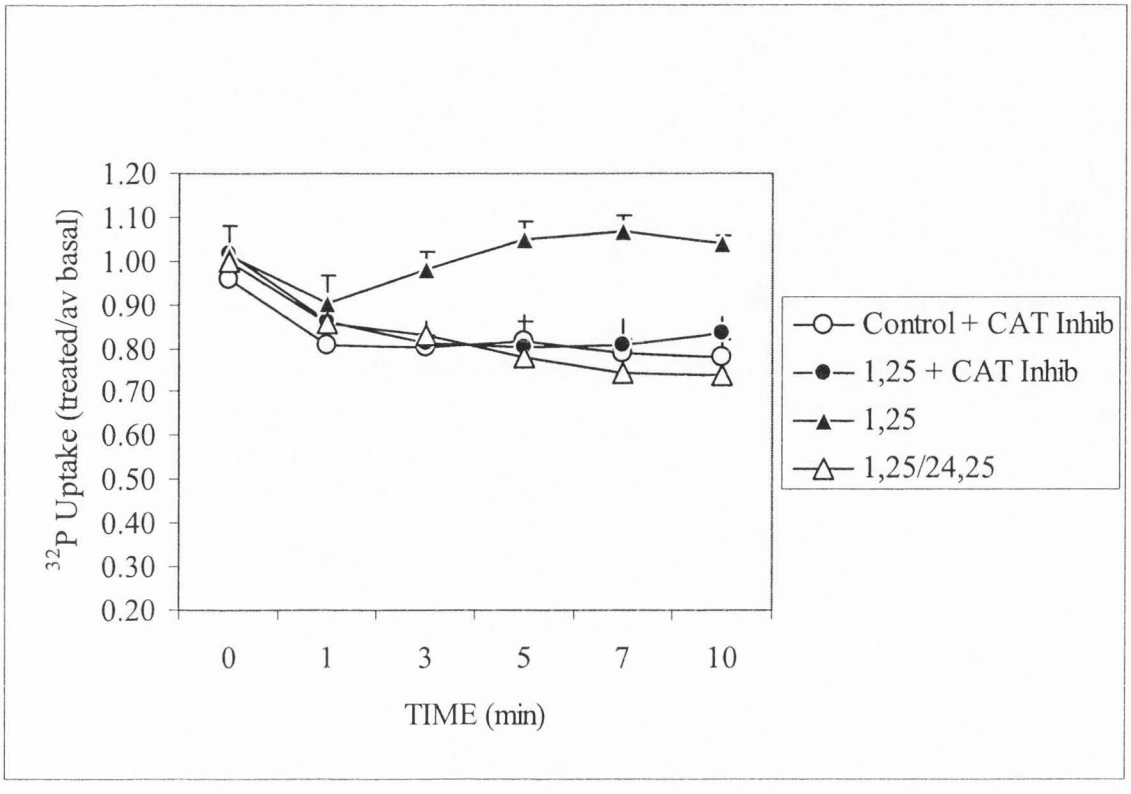

Fig. 4. Time course study of ^{32}P uptake and effect of catalase inhibitor in isolated intestinal cells. Enterocytes were isolated and exposed to radionuclide as described in the legend to Fig. 2. Aliquots of cell suspension were pipetted into four fresh tubes, two of which contained 1 μM catalase inhibitor (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 $24,25(OH)_2D_3$ (Δ—Δ; 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 (1000 x 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.

inhibition of $1,25(OH)_2 D_3$. 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 to10 min (P = 0.01-0.001), relative to corresponding controls or corresponding samples from cells treated with both steroids alone. At 10 min of incubation, cells treated with $1,25(OH)_2D_3$ accumulated radionuclide to 133 % of vehicle controls, while those exposed to catalase inhibitor or hormone alone exhibited ^{32}P levels that were less than $\pm$ 2% of vehicle controls.

^{32}P Uptake and 4β-Phorbol 12-myristate 13-acetate

Figure 5 illustrates the results of experiments designed to test whether the production of hydrogen peroxide mediated by $24,25(OH)_2D_3$ can act to inhibit PKC signaling independently of its action on the $1,25D_3$-MARRS receptor. In these studies addition of 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. 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. 5). Addition of PMA alone, resulted in enhanced levels of ^{32}P uptake that were evident within 1 min after addition, and which became statistically significant at 5 to10 min (P = 0.02-0.001), relative to corresponding controls or corresponding samples from cells treated with both hydrogen peroxide and PMA. At 10 min of incubation, cells treated with PMA accumulated radionuclide to 143 % of vehicle controls, while those exposed to hydrogen peroxide and PMA exhibited ^{32}P levels that were less than $\pm$ 2% of vehicle controls.

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

Earlier evidence [Nemere et al., 2006 manuscript submitted] 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 either phosphate uptake or 1,25$(OH)_2D_3$-stimulated PKC activity occurred within 1 min. And as shown in Fig. 5, direct stimulation of phosphate uptake with phorbol ester was rapidly inhibited by H_2O_2, suggesting that the oxidant might directly inhibit PKC activation. Experiments were therefore performed to assess this possibility.

Figure 6 illustrates the time course of PKC activation in response to treatment of isolated intestinal epithelial cells with 100 nM PMA in the absence or presence of 50 μM H_2O_2. Vehicle controls incubated in the presence of hydrogen peroxide showed a decrease in PKC levels with time (open circles). Stimulation of PKC activity was detected 1 minute after addition of PMA. Protein kinase C levels were noticeably greater in such cell suspension within 1 min, and became significantly greater than corresponding incubations with H_2O_2 at 3 to 5 min ($P<0.05$; Fig. 6). At 5 min of incubation, PMA-treated cells showed PKC levels 148 % of controls.
PKC activity was stimulated to a lesser degree by the addition of 1,25$(OH)_2D_3$.
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. 6).

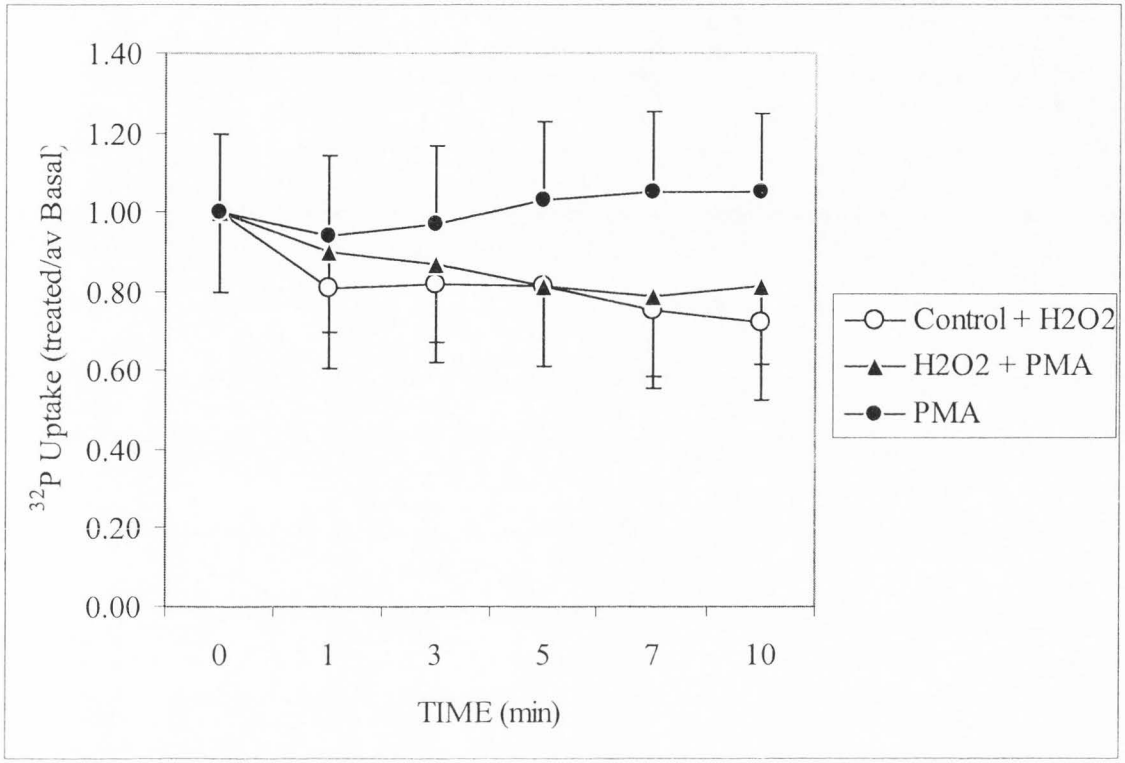

Fig. 5. Time course study of ^{32}P uptake and effect of 4β-Phorbol 12-myristate 13-acetate (PMA) in isolated intestinal cells. Enterocytes were isolated and exposed to radionuclide as described in the legend to Fig. 2. Aliquots of cell suspension were pipetted into three fresh tubes, two of which contained 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 (1000 x 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.

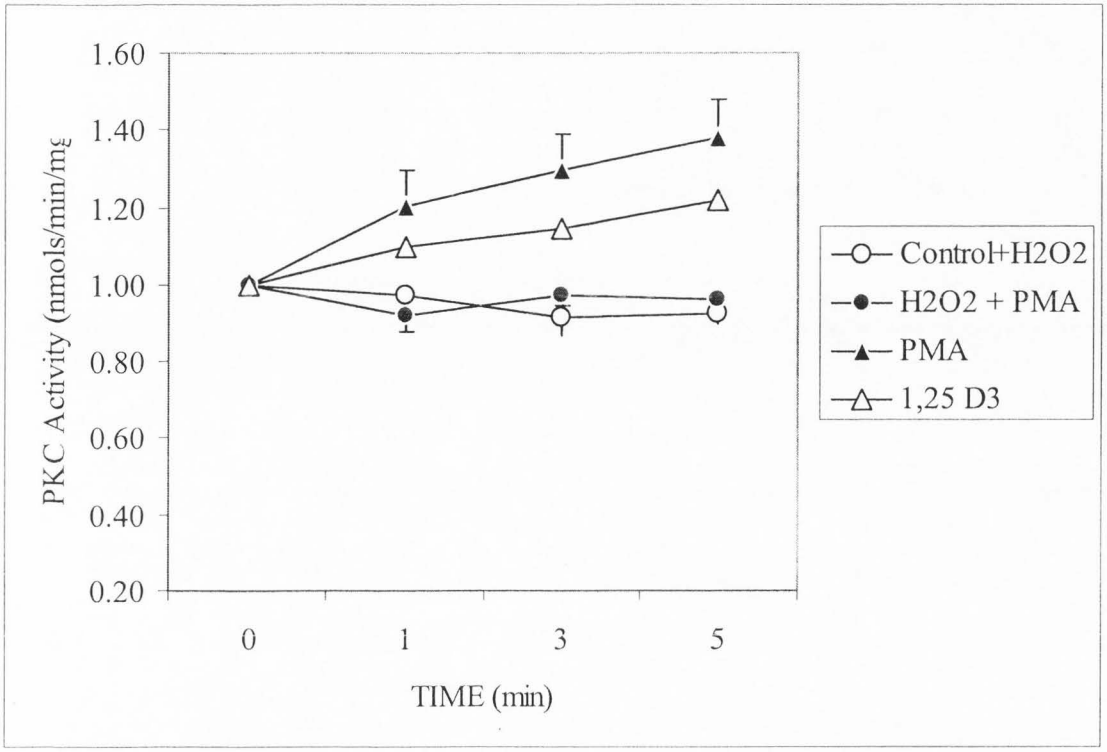

Fig. 6. Time course study of PKC activation by 4β-Phorbol 12-myristate 13-acetate (PMA) in isolated intestinal cells. At T = 0 min, the cells were treated with H_2O_2, PMA, or $1,25(OH)_2D_3$ to produce the following incubation conditions: (1) Control plus 50 μM H_2O_2 (○—○; n=3); (2) 50 μM H_2O_2 plus 100 nM PMA (●—●; n=3); (3) 100nM PMA (▲—▲; n=3); (4) 300 pM $1,25(OH)_2D_3$ (Δ—Δ; n=2). Cell suspensions were sampled at T=0, 1, 3, and 5 min. After incubation, as described above, the cells were centrifuged at 1000 x 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 activity.

CHAPTER IV

DISCUSSION

The current work considerablely increases our understanding of the vitamin D endocrine system through further explanation of the signal transduction pathway that allows $24,25(OH)_2D_3$ to inhibit the actions of $1,25(OH)_2D_3$.

In a previous report, a cellular binding protein for $24,25(OH)_2D_3$ was established to be the enzyme catalase [Larsson et al., 2006]. Treatment of intestinal cells with $24,25(OH)_2D_3$ was subsequently found to inhibit catalase activity and promote an increase in H_2O_2 formation [Nemere et al., 2006 manuscript submitted]. It was hypothesized that the H_2O_2 might, in turn, oxidize thiols within the $1,25D_3$-MARRS receptor to decrease binding of $1,25(OH)_2D_3$, and ultimately inhibit stimulated phosphate uptake.

In the present work, it was found that neutralization of the $24,25(OH)_2D_3$ binding protein with Ab 365 in cells treated with both $24,25(OH)_2D_3$ and $1,25(OH)_2D_3$ effectively reversed the inhibition. These results extend another work [Larsson et al., 2006] in which commercially available anti-catalase antibody was also found to reverse $24,25(OH)_2D_3$ inhibition of $1,25(OH)_2D_3$-stimulated phosphate uptake in isolated intestinal epithelial cells. Moreover, Ab 365 directed against a protein isolated on the basis of specific $[^3H]24,25(OH)_2D_3$ binding, was demonstrated to recognize bovine catalase by Western analysis [Larsson et al., 2006].

In the current study, the addition of exogenous catalase also reversed the $24,25(OH)_2D_3$-mediated inhibition of $1,25(OH)_2D_3$. Although whether this occurred

through the enzyme's catalytic activity or through sequestration of $24,25(OH)_2D_3$ was not determined.

In addition, catalase inhibitor reproduced the $24,25(OH)_2D_3$-mediated inhibition of $1,25(OH)_2 D_3$. This suggests that catalase has a central role in mediating rapid responses to steroid hormones.

In other work [Nemere et al., 2006 manuscript submitted], incubation of isolated intestinal epithelial cells with $24,25(OH)_2D_3$ resulted in a time-dependent decrease in the ability of the $1,25D_3$-MARRS receptor to bind its ligand. Decreased binding was not evident after 5 min of incubation, indicating two things: $24,25(OH)_2D_3$ does not compete with $1,25(OH)_2D_3$ for binding to the $1,25D_3$-MARRS receptor, as previously reported [Nemere et al., 1994]; and there must be an additional, more rapid mechanism to account for the inhibitory effects of $24,25(OH)_2D_3$. Thus, experiments were performed to assess the prospect of hydrogen peroxides ability to down regulate PKC activity. Phorbol ester was first demonstrated to stimulate phosphate uptake in isolated enterocytes, and enhanced uptake was found to be susceptible to inhibition by H_2O_2, strongly suggesting that PKC, the signal transduction pathway responsible for mediating phosphate uptake, was a target of oxidant activity. Finally, by measuring PKC activity in response to phorbol, it was found that H_2O_2 did directly abolish stimulation.

The combined research has implications at the organismal level. In agriculture, a serious problem with poultry farming is the production of manure high in phosphates. While beneficial for crop production, leaching of the phosphates into nearby waterways has had a damaging effect on ecology. It is likely that by increasing dietary antioxidants, phosphate absorption will be increased, with concomitant decreases in manure levels.

Beyond agriculture, these studies have implications for bone health in all animals, including humans. Diets enriched in antioxidants have been found to promote bone health [Lean et al., 2003]. In relation to this, Magne et al. [2003] have reported that the cellular signal for bone mineralization is increased phosphate. While phosphate is not a limiting nutrient, as calcium is, it is apparently subject to intricate regulation. Earlier work in this lab has demonstrated that the rapid stimulation of phosphate absorption through the $1,25D_3$-MARRS receptor is confined to growing animals [Nemere et al., 2004], which correlates with periods of rapid bone formation. It is moreover believed that by maximizing bone mineral density early in life, osteoporosis can be ameliorated. Clearly, a viable strategy for enhancing bone formation may be diets rich in antioxidants.

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APPENDICES

Appendix A. Abbreviations

μCi .....Microcurie, a term used to quantify radioactivity.

μg .....Micrograms.

μl .....Microliter.

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

$1,25D_3$-MARRS .....1,25-dihydroxyvitamin D_3 membrane-associated, rapid-response steroid-binding) protein.

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

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

^{32}P.....Radioisotope of phosphorous from abbreviated notation of $H_3{}^{32}PO_4$.

^{45}Ca .....Radioisotope of calcium.

Ab 365.....Antibody 365, used to block the $24,25(OH)_2D_3$ binding protein.

ANOVA .....Analysis of Variants.

BP.....Binding protein.

C.....Celsius.

Ca^{2+} .....Calcium.

CaBP .....Calcium binding protein, also termed $Calbindin_{28k}$.

cpm/μg .....Counts Per Minute over Micrograms of protein.

ELISA .....Enzyme-Linked Immunosorbent Assay.

g.....Gravity.

GBSS.....Gey's Balanced Salt Solution.

H_2O_2.....Hydrogen peroxide.

mM.....Millimolar.

mRNA.....Messenger RNA.

nM.....Nanomolar.

P .....P-value statistical measure.

PKA.....Protein Kinase A.

PKC.....Protein Kinase C.

pM.....Picomolar.

PMA.....4β-Phorbol 12-myristate 13-acetate.

SEM .....Standard Error of the Mean.

T .....Abbreviation of Time.

VDR .....Vitamin D Nuclear Receptor.

Appendix B. Statistical Analyses

Table A. ANOVA for figure 2. Time course of ^{32}P Uptake and effect of Ab 365 against the$24,25(OH)_2D_3$ binding protein.

Source of Variation	DF	SS	MS	F	P
Treatment	3	1.070	0.357	26.264	<0.001
Time	6	0.700	0.117	8.596	<0.001
Treatment x Time	18	0.605	0.0336	2.476	0.002
Residual	140	1.901	0.0136		
Total	167	4.369	0.0262		

Table B. ANOVA for figure 3. Time course study of ^{32}P uptake and effect of exogenous catalase in isolated intestinal cells.

Source of Variation	DF	SS	MS	F	P
Trt	3	0.528	0.176	11.562	<0.001
Time	6	0.501	0.0835	5.485	<0.001
Trt x Time	18	0.449	0.0249	1.637	0.062
Residual	112	1.705	0.0152		
Total	139	3.182	0.0229		

Table C. ANOVA for figure 4. Time course study of ^{32}P uptake and effect of catalase inhibitor in isolated intestinal cells.

Source of Variation	DF	SS	MS	F	P
Trt	3	0.598	0.199	15.732	<0.001
Time	6	0.572	0.0953	7.521	<0.001
Trt x Time	18	0.407	0.0226	1.786	0.035
Residual	112	1.419	0.0127		
Total	139	2.996	0.0216		

Table D. ANOVA for figure 5. Time course study of ^{32}P uptake and effect of 4β-Phorbol 12-myristate 13-acetate (PMA) in isolated intestinal cells.

Source of Variation	DF	SS	MS	F	P
conditions	2	0.298	0.149	10.865	<0.001
TIME	6	0.389	0.0648	4.721	<0.001
conditions x TIME	12	0.368	0.0307	2.234	0.027
Residual	42	0.577	0.0137		
Total	62	1.633	0.0263		

Table E. ANOVA for figure 6. Time course study of PKC activation by 4β-Phorbol 12-myristate 13-acetate (PMA) in isolated intestinal cells.

Source of Variation	DF	SS	MS	F	P
conditions	3	0.389	0.130	14.325	<0.001
TIME	3	0.0633	0.0211	2.330	0.013
conditions x TIME	9	0.161	0.0178	1.970	0.013
Residual	16	0.145	0.00905		
Total	31	0.758	0.0244		