Rapid Effects of 25-Hydroxyvitamin D$_3$ on Calcium Uptake in Isolated Chick Enterocytes

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RAPID EFFECTS OF 25-HYDROXYVITAMIN D₃ ON CALCIUM UPTAKE IN
CHICK ENTEROCYTES

by

Ruta R. Phadnis

A thesis submitted in partial fulfillment
of the requirements for the degree

of

MASTER OF SCIENCE

in

Nutrition and Food Sciences

Approved:

UTAH STATE UNIVERSITY
Logan, Utah
2003
ABSTRACT

Rapid Effects of 25-Hydroxyvitamin D₃ on Calcium Uptake in Isolated Chick Enterocytes

by

Ruta Phadnis, Master of Science
Utah State University, 2003

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25-Hydroxyvitamin D₃ [25(OH)D₃] is a metabolite of vitamin D₃ that has long been considered to be an inactive precursor of the hormonally active metabolite 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃]; consequently very few studies have addressed the potential biological activity of 25(OH)D₃. However, it is known that 100 nM 25(OH)D₃ increases calcium transport in the perfused duodenal loop of the chicken to 200% of controls within 20 minutes. The hypothesis of the current study is that 25(OH)D₃ may be a hormonally active metabolite and its effects can be studied in isolated chick enterocytes. To begin testing this postulate, time course studies of ⁴⁵Ca uptake were undertaken in isolated intestinal cells (from 7 wk chicks). After establishing the basal uptake of ⁴⁵Ca for 5 minutes, cells were treated with vehicle (< 0.01% v/v ethanol, final concentration) or 25 nM, 50 nM, 100 nM, or 300 nM 25(OH)D₃ and samples were taken at T = 1, 3, 5, 7, and 10 min. With increasing concentrations of steroid, the enterocyte
$^{45}$Ca decreased. The optimal concentration of 100 nM 25(OH)D$_3$ induced the most rapid response: within 1 min $^{45}$Ca decreased to 54% of controls ($P<0.001$) and 70% of the controls at T= 3, 5, and 7 min ($P<0.01$ to $<0.05$, relative to controls). Comparison of the 7-min time points for 25 nM, 50 nM, 100 nM, and 300 nM 25(OH)D$_3$ appeared to yield a biphasic dose response curve with values of $^{45}$Ca observed at 99% (NS, not significant), 75% ($P<0.05$), 70% ($P<0.01$%), and 80% (NS) of corresponding controls, respectively. Physiological levels of 24,25(OH)$_2$D$_3$ (6.5 nM) inhibited the action of 100 nM 25(OH)D$_3$ in isolated chick enterocytes. Time course studies with isolated enterocytes from 14 wk and 28 wk chickens treated with 100 nM 25(OH)D$_3$ also showed decreased responsiveness: at T=1 min $^{45}$Ca levels in 7 wk, 14 wk, and 28 wk were 54% ($P<0.01$), 83% (NS), and 80% (NS) of corresponding controls, respectively. Experiments with the calcium channel activator BAY K8644 (2 µM) and protein kinase A (PKA) activator forskolin (20 µM) revealed enhanced levels of $^{45}$Ca at T=10 min that were 132% and 140% of corresponding controls, respectively (each, $P<0.05$). Phorbol ester treatment of the cells resulted in significant increases in the levels of $^{45}$Ca between the treated cells and corresponding controls at T=7 and 10 min. Cells treated with 100 nM 25(OH)D$_3$ revealed 89.8% and 78.4% increases above controls in PKA activity at T =1 min ($P<0.05$) and T=3 min, relative to corresponding controls. However, there was no evidence for the activation of PKC by 25(OH)D$_3$ during the time period studied.
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Ruta R. Phadnis
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CHAPTER I
INTRODUCTION

Pochon and De Luca (1969) first reported that vitamin D is mostly found in the form of 25-hydroxyvitamin D$_3$ [25(OH)D$_3$] in the body, indicating that it is a major metabolite of vitamin D$_3$. The liver is the predominant site of 25-hydroxylation of vitamin D but not the exclusive site (Ponchon and De Luca, 1969; Olson et al., 1976).

The earliest studies on 25(OH)D$_3$ reported its activities on vitamin D target organs at pharmacologic levels (Pavlovitch et al., 1973), while Boyle et al. (1972) and Holick et al. (1972) reported that the metabolite was inactive at physiological concentrations, and required conversion to an active hormonal form subsequently identified as 1,25 hydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$] which is produced in the kidney.

However, it has also been found that 25(OH)D$_3$ is 2-5 times more active than the parent vitamin D$_3$ in all systems known to respond to vitamin D (Blunt et al., 1968; Tanaka and DeLuca, 1973). A number of researchers have provided evidence for the actions of 25(OH)D$_3$ independent of metabolism to 1,25(OH)$_2$D$_3$ (Nemere et al., 1984; Yoshimoto and Norman, 1986; Gacad et al., 1996; Nemere, 1996a; Teegarden et al., 1997, 2000; Wu et al., 2000) in a variety of biological systems.

The vitamin D endocrine system acts through both long term genomic and membrane initiated actions (Nemere, 1996b). One of the principle target organs of the most studied vitamin D$_3$ metabolite, 1,25(OH)$_2$D$_3$, is the intestine. The perfused chick duodenal loop has been a valuable system for the study of rapid intestinal calcium and phosphate transport (Nemere et al., 1984, 1986). The action of vitamin D metabolites on
calcium homeostasis has been studied in isolated intestinal cells as well (Nemere and Szego, 1981a,b).

Another renal metabolite of 25(OH)_{2}D_{3}, 24,25-hydroxyvitamin D_{3} \{24,25(OH)_{2}D_{3}\}, which was once considered to be an inactive metabolite of vitamin D_{3}, is now known to suppress the actions of 1,25(OH)_{2}D_{3} on calcium and phosphate transport in the chick intestine (Nemere, 1999), and to inhibit Ca^{2+} channel opening in osteoblasts (Takeuchi and Guggino, 1996).

The specific objectives of this research were to determine the possible biological activity of 25(OH)D_{3} on calcium uptake in chick intestinal cells, to identify, if possible, the signal transduction pathways associated with the activity, and to study the effects of 24,25(OH)_{2}D_{3} and aging on the response generated by 25(OH)D_{3}.

1,25(OH)_{2}D_{3} Membrane Initiated Signalling

A brief summary of both membrane initiated actions of vitamin D metabolites in stimulating the calcium transport pathway and the recent developments in 25(OH)D_{3} research are necessary to discuss the results of this project. Nemere and Szego (1981a,b) first observed the pre-nuclear effects of 1,25(OH)_{2}D_{3} in isolated rat intestinal cells, and the authors postulated that the presence of a membrane localized receptor for the hormone might be responsible for this action. Lieberherr et al. (1989) also noted the need for a membrane receptor after observing 1,25(OH)_{2}D_{3} mediated rapid changes in phosphoinositide metabolism. Wali et al. (1990, 1992) and Khare et al. (1994) reported protein kinase C activation in the rapid effects of 1,25(OH)_{2}D_{3}. Work by Nemere and Norman (1987) supported the idea of non-nuclear actions for vitamin D metabolites when
they reported the inability of actinomycin to inhibit 1,25(OH)₂D₃ augmented calcium transport in the perfused chick duodenum. de Boland et al., (1990) reported opening of calcium channels in the basal lateral membranes as a result of ligand binding to the membrane receptors.

Both Bay K8644 (de Boland et al., 1990) and phorbol 12-myristate 13- acetate, (PMA, de Boland et al., 1990) can mimic the action of 1,25(OH)₂D₃ in the intestine. Nemere et al. (1994) have also reported the presence of an integral membrane protein in the basal lateral membrane of the intestinal cell for which 1,25(OH)₂D₃ has a high affinity. The ligand binding to the putative receptor is saturable.

Studies on 25(OH)D₃

Studies by Heaney et al. (1977, 1989) and Bell et al. (1988) indicate that 25(OH)D₃ may have a biological role in regulating cell growth and calcium transport. In addition, Gacad et al. (1996) have reported functional characterization and purification of an intracellular vitamin D-binding protein (IDBP) in vitamin D-resistant New World primates. This 60-65 kD protein allows binding of 25-hydroxylated vitamin D metabolites only. Specifically, hydroxylation at C-1 diminished but did not abolish ligand binding. Wu et al. (2000) have also shown that IDBP along with bound 25(OH)D₃ has transcriptional effects which demonstrates that the metabolite has biological activities independent of 1,25(OH)₂D₃. Teegarden et al. (1997) purified and partially characterized a unique protein in rat enterocytes which can bind to 25(OH)D₃. It has been postulated by the authors that this cellular binding protein might have a role in mediating the effects of 25(OH)D₃ within the enterocytes. Teegarden et al. (2000) also reported that this steroid
binding protein (from Caco-2 cells) binds to 25(OH)D₃ with 1000-fold greater affinity than to 1,25(OH)₂D₃ or 24,25(OH)₂D₃.
Background

Although vitamin D was discovered as a nutritional factor in 1922 by McCollum and co-workers, it is considered a pro-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. Since under these conditions, vitamin D is not required in the diet, it does not meet the definition of a vitamin. Moreover, it is further metabolized into more active forms, which is a property of a pro-hormone.

Vitamin D is metabolized in the body to 25(OH)D₃, which in turn is transformed into more polar metabolites: 1,25(OH)₂D₃, a hormonally active secosteroid and 24,25(OH)₂D₃, 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).

Vitamin D is the name applied to two fat-soluble substances -- cholecalciferol and ergocalciferol. Cholecalciferol is without biological activity at physiological concentrations. For activation, cholecalciferol must first be converted into 25(OH)D₃ in the liver. The liver appears to be the major site of 25-hydroxylation in mammals. In the chicken however, the 25 hydroxylase enzyme exists in extra-hepatic sites such as the kidney and the intestines. It is generally accepted that although this steroid is a major circulating metabolite of cholecalciferol it must undergo chemical modification to 1,25(OH)₂D₃ or 24,25(OH)₂D₃ before it can function as a hormone.
A well-studied vitamin D metabolite target organ is the intestine. In the intestine, the nuclear receptor of the metabolite, \( 1,25(OH)_2D_3 \), may mediate 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 mediates the CaBP synthesis.

**Mechanisms of Biological Action of Vitamin D Metabolites**

The most commonly studied metabolite of vitamin D is \( 1,25(OH)_2D_3 \), and it has been shown that this metabolite acts both by genomic and membrane-initiated actions (Nemere, 1996b).

Various genomic 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 lysosomes. Warner and Coleman (1975) using X-ray probe analysis in conjunction with electron microscopy, 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 \(^{45}\text{Ca} \) were found in lysosomal fractions after transport. Time course (Nemere and Norman, 1988) and dose-response studies (Nemere, 1999) further supported the vesicular carrier hypothesis.
Wasserman and Taylor (1968) reported the discovery of a high affinity calcium binding protein in the chick mucosa following vitamin D repletion of deficient birds. The 28kD protein, whether induced by 1,25(OH)_{2}D_{3} or induced as a consequence of hormone-enhanced calcium transport, has been postulated either to be the calcium carrier in the intestinal epithelial cells or to serve as a buffer protein to prevent the deleterious effects associated with high intracellular calcium. In two separate studies Nemere et al. (1986, 1991) reported that lysosomes were found to be enriched in calbindin-D_{28k}. Using ELISA in conjugation with subcellular fractionation studies, lysosomal fractions were found to be enriched in calbindin-D_{28k}, although most of the protein remained soluble. 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 increasing times after 1,25(OH)_{2}D_{3} treatment resulted in decreasing levels of message but increasing levels of protein. The inverse relation between α-tubulin and its message has been ascribed to autoregulation (Cleveland and Sullivan, 1985).

Cai et al. (1993) have observed a further genomic effect of vitamin D in that the hormonally active metabolite increases mRNA of the calcium ATPase. The enzyme is located in the basal lateral membrane of the intestinal epithelial cells and may function to decrease free cytoplasmic levels. It is possible that this pump may serve to terminate
signal transduction events initiated during the non-genomic actions of 1,25(OH)₂D₃ (Nemere, 1996b).

Membrane Initiated Actions

It can be argued that when an animal reaches a Ca²⁺ deficient state, it would be beneficial to obtain crucial Ca²⁺ rapidly from the diet without delay due to transcription. Given the pivotal role of Ca²⁺ 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 some 25 years ago (Toffolon et al., 1975). Toffolon et al. (1975) reported that within 30 mins of 1,25(OH)₂D₃ 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 bi-layer of the apical membrane of the intestinal cells and thereby exert a direct membrane effect.

A direct rapid effect of 1,25(OH)₂D₃ 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 rapid effects observed (Nemere and Szego, 1981a,b). In 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)₂D₃ stimulated phosphoinositide metabolism (apparent within 5 sec of hormone treatment). Further downstream of the initial phosphoinositide turnover mediated by 1,25(OH)₂D₃, protein kinase C 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)_{2}D_{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)_{2}D_{3} however, decreased Ca^{2+} transport in the perfused intestine of the Atlantic cod, a marine fish (Sundell et al., 1990; Larsson et al., 1995).

The perfused chick duodenal loop is a valuable system for the study of rapid actions of 1,25(OH)_{2}D_{3} and 24,25(OH)_{2}D_{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)_{2}D_{3} acts at the basal lateral membrane (BLM) surface and not at the brush border (Nemere et al., 1984, 1996a,b) this observation suggests binding to a membrane receptor, rather than non-specific membrane insertion by 1,25(OH)_{2}D_{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.

Other Factors Affecting Ion Transport

The metabolite 24,25(OH)_{2}D_{3}, is preferentially produced when an animal is calcium-, phosphate- and 1,25(OH)_{2}D_{3}- replete. Thus, it may be an endogenous inhibitor of 1,25(OH)_{2}D_{3}. In this regard, it has been shown that 24,25(OH)_{2}D_{3} suppresses the rapid actions of 1,25(OH)_{2}D_{3} on phosphate and calcium transport in the chick intestine (Nemere, 1996a,1999). At a cellular level 24,25(OH)_{2}D_{3} has been found to inhibit the rapid membrane initiated effects of 1,25(OH)_{2}D_{3} on opening of calcium channels in osteoblasts and osteosarcoma cells (Yukhiro et al., 1994; Khoury et al., 1995; Takeuchi and Guggino, 1996) and PKC activation in chick enterocytes (Nemere, 1999).
It is known that intestinal calcium absorption and adaptation to dietary calcium restriction decrease with age in humans and rats. A study conducted by Armbrecht et al. (1980) found out that exogenous 1,25(OH)$_2$D$_3$ but not 25(OH)D$_3$ could partly overcome the age dependent decrease in absorption.

Evidence for the Biological Effect of 25(OH)D$_3$

Olson and DeLuca (1969) reported that addition of 25(OH)D$_3$ to vitamin D deficient rat intestine via arterial perfusate induced a rise in calcium transport to vitamin D replete levels within 2 hours. In contrast it was also reported in this study that vitamin D$_3$ given in the same manner (and in higher concentration) had no effect on the Ca$^{2+}$ transport over a 4-hour period. This could be interpreted to indicate that 25(OH)D$_3$ represents a metabolically active form of vitamin D$_3$, although it cannot be ruled out that during the 2 hour period, 25(OH)D$_3$ was further metabolized to 1,25(OH)$_2$D$_3$.

Another study by Yoshimoto and Norman (1986) reported a comparison of vitamin D$_3$, its derivatives and analogs and their ability to promote $^{45}$Ca transport in an isolated duodenal loop perfusion system of the vitamin D-replete chick. It was seen that 25(OH)D$_3$ caused an onset of significant increment in the level of $^{45}$Ca transport in 8 mins (minimum effective concentration, 6.5nM steroid). The premise of endocrine action is the evocation of physiological response as a consequence of hormone binding to a specific receptor. While steroid hormones often act through a specific receptor to alter gene expression, the rapidity of the response to 25(OH)D$_3$ in the chick model system (Nemere et al., 1984; Nemere, 1996a) suggests the vitamin D metabolite may activate membrane associated signal transduction systems. Teegarden et al. (1997, 2000) have also reported isolation and characterization of a 25(OH)D$_3$ binding protein from intestinal
expression, the rapidity of the response to 25(OH)D₃ in the chick model system (Nemere et al., 1984; Nemere, 1996a) suggests the vitamin D metabolite may activate membrane associated signal transduction systems. Teegarden et al. (1997, 2000) have also reported isolation and characterization of a 25(OH)D₃ binding protein from intestinal cells which binds to 25(OH)D₃ with at least 1000-fold greater affinity than 1,25(OH)₂D₃ or 24,25(OH)₂D₃. It has been suggested that this protein might have a role in regulating the signaling pathway of 25(OH)D₃ within cells.

My hypothesis is that 25(OH)D₃ may be a hormonally active form of vitamin D, and its effects can be studied in isolated enterocytes. The objectives of this research were:

1. To study ⁴⁵Ca uptake in isolated chick intestinal cells as influenced by 25(OH)D₃. This was accomplished through time courses with a range of 25(OH)D₃ concentrations. In order for a response to be considered physiologically relevant it must occur at or near circulating levels of 25(OH)D₃.

2. To determine whether 24,25(OH)₂D₃ affects 25(OH)D₃ mediated uptake of ⁴⁵Ca by isolated intestinal cells. 24,25(OH)₂D₃ appears to be an endogenous inhibitor of the stimulatory action of 1,25(OH)₂D₃ (Nemere, 1999). Therefore it would be useful to assess the potential interaction between 25(OH)D₃ and 24,25(OH)₂D₃.

3. To analyze the effect of age on the action of 25(OH)D₃ with respect to ⁴⁵Ca absorption in the chick intestinal cell.

4. To study the signal transduction pathways that might be involved in the 25(OH)D₃ mediated ⁴⁵Ca uptake; in particular protein kinases A and C.
CHAPTER III
MATERIALS AND METHODS

Animals and Surgical Procedures

All of the procedures were approved by Utah State University Institutional Animal Use and Care Committee. White leghorn cockerels were obtained on the day of hatch (Merill Poultry, Poul, ID) and raised on a vitamin D-replete diet (Nutrena Feeds, Murray, UT) generally for 3-7 wks prior to experimentation. On the day of use, chicks were anesthetized with 0.3 ml chloropent/100g 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 [isolation medium first described by Weiser (1973), 96 mM NaCl, 1.5 mM KCl, 8 mM KH$_2$PO$_4$, 5.6 mM Na$_2$HPO$_4$, 27 mM sodium citrate, pH 7.0] 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 40 ml of Gey's Balanced Salt Solution [GBSS, containing 119 mM NaCl, 4.96 mM KCl, 0.22 mM KH₂PO₄, 1.03 MgCl₂·6H₂O, 0.28 mM MgSO₄·7H₂O, 0.89 mM CaCl₂, buffered to pH 7.4 and with 0.1% bovine serum albumin, (BSA) added] by gentle dispersion with a Teflon coated rod to avoid breaking the fragile intestinal cells followed by dropwise addition of GBSS-BSA.

**⁴²Ca Uptake In vitro**

Five ml of the cell suspension in GBSS was removed to a polypropylene tube (Falcon, Fischer Scientific) containing 5 µCi of ⁴⁵CaCl₂ (Perkin Elmer Life Sciences, Boston, MA). After gently mixing, duplicate 2 ml aliquots were removed to two separate fresh tubes for time course studies.

Duplicate 100 µl aliquots were removed at T = -5 min and -1 min to establish basal uptake rates of ⁴⁵Ca. At T = 0 min, the cells were treated with the vehicle ethanol (< 0.01% final concentration) or a range of 25(OH)D₃ concentration (25 nM, 50 nM, 100 nM or 300 nM metabolite). At T = 1, 3, 5, 7, and 10 min duplicate 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 10 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 1 ml of double-distilled water, and aliquots taken for liquid scintillation spectrophotometry and protein determination.
Effect of Age on 25(OH)D₃ Mediated ⁴⁵Ca Uptake in Enterocytes

Using the optimal concentration of 25(OH)D₃ determined in the dose response studies, time course experiments were conducted with male chickens at 14 and 28 wks of age.

Effect of 24,25(OH)₂D₃ on 25(OH)D₃ Mediated ⁴⁵Ca Uptake in Enterocytes

With enterocytes isolated from 7 wk chicks time course studies similar to those described above were undertaken with an optimal concentration of 25(OH)D₃ (determined in the dose response studies) in the presence of 6.5 nM 24,25(OH)₂D₃ and compared to vehicle controls.

Effect of Signal Transduction Agonists on ⁴⁵Ca Transport Using Time Course Studies

Isolated intestinal cells were treated with 20 µM forskolin (a protein kinase A agonist), 100 nM 12-0-tetradecanoyl-13 phorbol acetate, TPA an activator of protein kinase C), or with 2 µM BAY K8644, a calcium channel activator. Controls received the vehicle dimethyl sulfoxide (DMSO) at 0.2%, v/v final concentration.

Effect of 25(OH)D₃ on Protein Kinase A and C Activities

Isolated intestinal cells were treated with 100 nM 25(OH)D₃ or the vehicle ethanol (< 0.01% v/v, final concentration) and duplicate 100 µl samples were taken at T = 1, 3, 5, 7, and 10 min after addition of the hormone or vehicle. To establish basal values,
duplicate 100 µl samples were also drawn at T = -5 min and -1 min. Each sample was pipetted into 900 µl of GBSS and centrifuged at 1000 x g for 10 min.

*Analytical Determinations*

Protein Kinase C Assay: The harvested cell pellets were homogenized with 0.5 ml extraction buffer (buffer A, 20 mM Tris, pH 7.5, 0.5 mM EGTA, 0.5% Triton-X 100, 25 µg/ml aprotinin and leupeptin) with 10-15 strokes on ice in a pre-cooled Dounce homogenizer. The homogenized cell solution was incubated for 30 min on ice. The cellular debris was then removed by centrifugation for 2 min (at 14,000 rpm) in a F3602 rotor (Beckman Instruments, Fullerton, CA). The supernatant was saved on ice, its volume measured, and a 100 µl aliquot was taken for protein determination. The protein concentration in the sample was then adjusted to 1 µg/µl. The assay and pre-incubation conditions were as given in Table 1.

All tubes were incubated for 20 min at room temperature to allow inhibitor to bind, prior to addition of 10 µl of 32P/substrate solution (20-25 µCi/ml of 5X PKC substrate solution) per tube. The contents were mixed and then incubated at 30°C for 5 min. At the end of this time, 25 µl of the mixture from each tube was removed and spotted onto corresponding phosphocellulose discs. The phosphocellulose discs were washed twice with 0.1% v/v phosphoric acid and twice with water. Peptide incorporated 32P/phosphocellulose disc was then measured using liquid scintillation spectrophotometry. Data were analyzed according to the instructions received with the Protein Kinase C Assay System (Gibco BRL, Life Technologies).
Protein Kinase A Assay: The harvested cell pellets were homogenized with 10-15 strokes in a glass Dounce homogenizer along with 0.5 ml extraction buffer (5mM EDTA, 50 mM Tris, pH 7.5). The cellular debris was removed as described above. The volume of the supernatant was measured and saved on ice. The protein concentration was adjusted to 1µg/µl using diluent (50mM Tris, pH 7.5). The assay and pre-incubation conditions were as per Table 2.

Following a 20 min pre-incubation at 23 C 10 µl of 32P/substrate solution (200 µM kemptide, 400 µM ATP, 40 nM MgCl2, 1 mg/ml BSA, 50 mM Tris, pH 7.5) containing 20-25 µCi/ml of radionuclide was added to each tube, and the tubes incubated at 30 C for 5 min. Twenty µl of the mixture was removed from each tube and spotted onto a labeled phosphocellulose disc. The phosphocellulose discs were then washed twice with 0.1% v/v phosphoric acid and twice with water. Peptide - incorporated 32P was counted using liquid scintillation spectrophotometry. Data were analyzed as per instructions received with the Protein Kinase A (cAMP-Dependent Protein Kinase) Assay System (Gibco BRL, Life Technologies).

Protein was determined using the Bradford dye from Bio-Rad (Hercules, CA, USA.

Statistical Analyses

Results are expressed as mean ± SE and as ratios of 45Ca in cells at time T divided by the basal 45Ca levels (average of 45Ca in cells of time T= 0 and T= -1). The significance (set at P<0.05) was determined by Students t-test for unpaired observations.
Table 1. Assay conditions for the determination of protein kinase C in the absence and presence of a specific enzyme inhibitor.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Enzyme</th>
<th>Elution Buffer</th>
<th>Inhibitor</th>
<th>Lipids</th>
<th>Water</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>5-25 µl</td>
<td>0-20 µl*</td>
<td>0 µl</td>
<td>5 µl</td>
<td>10 µl</td>
</tr>
<tr>
<td>B</td>
<td>5-25 µl</td>
<td>0-20 µl*</td>
<td>10 µl</td>
<td>0 µl</td>
<td>5 µl</td>
</tr>
</tbody>
</table>

Enzyme + Elution Buffer = 25 µl

Elution Buffer = 20 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM EGTA, 10 mM β-mercaptoethanol, 0.2 M NaCl).

Table 2. Assay conditions for the determination of protein kinase A activity in the absence and presence of a specific inhibitor.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Extract</th>
<th>Diluent</th>
<th>4X PKA Inhibitor</th>
<th>4X PKA Activator</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>10 µl</td>
<td>20 µl</td>
<td>0 µl</td>
<td>0 µl</td>
</tr>
<tr>
<td>b</td>
<td>10 µl</td>
<td>10 µl</td>
<td>10 µl</td>
<td>0 µl</td>
</tr>
<tr>
<td>b</td>
<td>10 µl</td>
<td>10 µl</td>
<td>0 µl</td>
<td>10 µl</td>
</tr>
<tr>
<td>d</td>
<td>10 µl</td>
<td>0 µl</td>
<td>10 µl</td>
<td>10 µl</td>
</tr>
</tbody>
</table>

Tube a* activity in sample
Tube c** total activity in sample
Tube b,d Equivalent to a and c, respectively but in presence of a specific inhibitor

4X PKA activator (4X = 40 µM cAMP, 50 mM Tris, pH 7.5)
4X PKA inhibitor (4X = 4 µM PKI (6-22) amide, 50 mM Tris pH 7.5)
CHAPTER IV
RESULTS AND DISCUSSION

Time Course of \(^{45}\)Ca Uptake after
\(25(OH)D_3\) In Vitro

Earlier studies have demonstrated that a physiological level of seco steroid, 100 nM \(25(OH)D_3\) preferentially stimulates calcium transport in the perfused duodenal loop, but not phosphate transport (Nemere, 1996a,b). In the current work, experiments were undertaken to investigate whether \(25(OH)D_3\) directly altered the calcium handling in isolated intestinal cells. In the time course studies, isolated enterocytes were treated with 1 µCi \(^{45}\)CaCl\(_2\)/ml of cell suspension and sampled at two points prior to addition of a range of \(25(OH)D_3\) concentrations or the vehicle ethanol (<0.01% v/v final concentration). The results of these studies are shown in Figs. 1-4.

Figure 1 shows that exposure of the cells to 25 nM \(25(OH)D_3\) elicited no change in \(^{45}\)Ca uptake relative to the controls. In the controls, the corresponding \(^{45}\)Ca treated/average basal values (mean ± SEM) for T= -1, -5 1, 3, 5, 7, and 10 min were 0.915 ± 0.045, 0.875 ± 0.060, 0.905 ± 0.045, 0.875 ± 0.060, 0.905 ± 0.079, 0.818 ± 0.049, and 0.763 ± 0.053 (n=32). The \(^{45}\)Ca treated/average basal levels (mean ± SEM) prior to addition were 1.14 ± 0.13, 0.86 ± 0.13 while at 1, 3, 5, 7, and 10 min after addition of the steroid in treated cells were 0.905 ± 0.01, 0.934 ± 0.11, 0.962 ± 0.04, 0.814 ± 0.01, 0.660 ± 0.05, respectively (n=4, P>0.05 at all time points).
Fig. 1. $^{45}$Ca uptake in enterocytes treated with 25 nM 25(OH)D$_3$ or the vehicle ethanol (<0.01% v/v final concentration). Cells isolated by the citrate chelation protocol were exposed to 1 µCi/ml of $^{45}$CaCl$_2$ at the beginning of the incubation (25°C). Duplicate 100 µl aliquots were removed at $T= -5$ and -1 to establish basal values. Addition of the steroid (closed symbols) or vehicle (open symbols) occurred at $T= 0$. Additional duplicate aliquots were taken at $T= 1, 3, 5, 7$, and $10$ min. Each aliquot was added to 900 µl of GBSS and centrifuged for 10 minutes at 1000 X g (4°C). The supernatants were decanted, the insides of the tubes swabbed and then the cell pellets were resuspended in 1 ml of distilled water for determination of protein and radionuclide activity. Values represent mean ± SEM for four independent experiments.
Figure 2 illustrates the results of experiments in which isolated enterocytes were treated with vehicle (open symbols) or 50 nM 25(OH)D₃ (closed symbols), a concentration of the steroid that is at the low end of circulating levels (Horst et al., 1981). It can be seen that for both the treatment groups there is a gradual decline in ^{45}Ca/mg protein with time. It is not known whether this is due to greater extrusion relative to uptake in these cells or whether cellular energy for uptake decreased during the incubation period, or if it is result of the incubation system. However, it is evident that 50 nM 25(OH)D₃ promoted a further decline in the cellular ^{45}Ca levels to 92% and 82% of corresponding controls (both, P<0.05), at T= 5 and 7 min, respectively (Fig. 2).

Treatment of cells with 100 nM seco steroid elicited a response that was distinctly different from those of the controls as early as one minute after addition of test substances (Fig. 3). As illustrated in this figure, the ^{45}Ca levels of the treated cells were much lower than those of the controls, at all time points after addition of the steroid. When expressed as a percent of control the ^{45}Ca values in treated cells at T=1, 3, 5, 7, and 10 min were 54% (P<0.01), 73% (P<0.05), 70% (P< 0.05), 71% (P<0.05), and 80% (P>0.05), respectively.

At 300 nM steroid, the highest concentration of 25(OH)D₃ tested, no significant change from the controls (P> 0.05) was observed in the treated cells at any of the time points tested (Fig. 4). The ^{45}Ca treated/ average basal levels (mean ± SEM) of the treated cells were approximately 80% of the controls at all time points observed after addition of the steroid. Figure 5 summarizes the results using the 7-min time point to highlight the apparent biphasic dose response curve of 25(OH)D₃ in the isolated chick enterocytes with
Fig. 2. $^{45}$Ca uptake in enterocytes treated with 50 nM 25(OH)D$_3$ or the vehicle ethanol (<0.01% v/v, final concentration). Cells were isolated, incubated, sampled, and processed as described in Fig. 1. Values represent mean ± SEM for six independent experiments. Statistical analyses were performed using Student’s t-test for paired observations.

* P<0.05 with respect to corresponding control value
Fig. 3. $^{45}$Ca uptake in enterocytes treated with 100 nM 25(OH)D$_3$ or the vehicle ethanol (<0.01% v/v, final concentration). Cells were isolated, incubated, sampled and processed as described in Fig. 1. Values represent mean ± SEM for 10 independent experiments. Significant differences, relative to corresponding controls were:

* $P<0.05$, relative to corresponding control value

** $P<0.01$, relative to corresponding control value
Fig. 4. $^{45}$Ca uptake in enterocytes treated with 300 nM 25(OH)D$_3$ or the vehicle ethanol (<0.01% v/v, final concentration). Cells were isolated, incubated, sampled, and processed as described in Fig. 1. Values represent mean ± SEM for 10 independent experiments.
Fig. 5. Summary of calcium transport at 7 min after hormone or vehicle addition. Data from Figs. 1-4 are replotted to allow for comparison of calcium uptake in isolated enterocytes due to increasing concentrations of 25(OH)D$_3$.

* P<0.05, relative to corresponding control value

** P≤0.01, relative to corresponding control value
respect to calcium handling. The data presented in this fashion clearly indicate that 100 nM 25(OH)D₃ was the optimal concentration of the steroid, and this was used in subsequent experiments.

*Effect of 24, 25(OH)₂ D₃ on the Action of 25(OH)D₃ with Respect to $^{45}$Ca Movement in Isolated Enterocytes*

Yoshimoto et al. (1986) and Nemere (1996c) have reported that physiological levels of 10 nM 24,25(OH)₂D₃ have a minimal effect on calcium or phosphate transport compared to duodena perfused with control media. Thus, experiments were undertaken to determine whether 24,25(OH)₂D₃ altered the previously observed 25(OH)D₃ stimulated response of calcium handling in isolated intestinal epithelial cells. A single physiological concentration of 6.5 nM 24,25(OH)₂D₃ (Horst et al., 1981) was tested in combination with 100 nM 25(OH)D₃ and compared to the vehicle controls (Fig. 6.) An apparent suppression of the effects of 25(OH)D₃ was observed. When expressed as a percent of control, $^{45}$Ca values (mean ± SEM) at T=1, 3, 5, 7 and 10 min after addition of the combined steroids were 119%, 101%, 101%, 98%, and 96%, respectively. The inhibitory effect of 24,25(OH)₂D₃ on 25(OH)D₃ stimulated calcium uptake was evident within 1 min after addition of the steroids. The degree of inhibition was evident at all time points and there was no significant difference between the levels of controls and those of the treated cells (P > 0.05 at all time points).

*Effect of Age on 25(OH)D₃ Stimulated Calcium Handling*
There is a gradual decrease in the intestinal calcium absorption with age in humans and animals (Avioli et al., 1965; Bullamore 1970), especially at low levels of dietary calcium (Ireland 1973 and Armbrecht et al., 1980). In the current study, experiments were conducted to compare whether age affected the 25(OH)D₃ stimulated calcium handling in isolated enterocytes. Time course studies as described above, were undertaken in which isolated enterocytes from 7 wk, 14 wk, and 28 wk old male chickens were treated either 100 nM 25(OH)D₃ or the vehicle ethanol (<0.1% v/v final concentration). The results of these studies are shown in Fig. 7. For comparison, the results from enterocytes of 7 wk chicks are shown in Fig. 7A. Cells treated with 25(OH)D₃ had ⁴⁵Ca levels that were 53.9%, 72.4%, 69.9%, 70.6%, and 12.4% of corresponding control values. Results of experiments in which isolated intestinal cells from 14 wk old chickens were treated with 100 nM 25(OH)D₃ showed a markedly decreased response compared to that obtained from 7 wk old chickens (Fig. 7B). Although the ⁴⁵Ca levels in the treated cells were lower than the controls at all time points after addition of the steroid, the differences were not statistically significant. The values of ⁴⁵Ca expressed as a percent of controls in cells from 14 wk chickens were 98%, 93%, 100%, 86%, and 76% at T = 1, 3, 5, 7 and 10 min, respectively (all P>0.05). Treatment of intestinal cells from 28 wk old chickens with 100 nM 25(OH)D₃ likewise showed a decrease in magnitude of response compared to younger chickens. Cells treated with 100nM 25(OH)D₃ yielded ⁴⁵Ca values that were 82%, 84%, 98%, 85%, and 76% of the controls, respectively. The differences between the treated and controls cells was not significant (P>0.05).
Results of Experiments with Signal Transduction Agonists

BAYK 8644

Time course studies were carried out in which isolated enterocytes were treated as described above for the addition of 2 µM BAYK 8644 (de Boland et al., 1990) or the vehicle DMSO. As seen in Fig. 8, the $^{45}\text{Ca}$ uptake in the BAY K8644 treated cells was much higher than that of controls after addition of the calcium channel activator. The $^{45}\text{Ca}$ levels at T=1, 3, 5, 7, and 10 min were 121%, 141%, 146%, 129%, and 132% of the corresponding controls, respectively. Statistical analysis of the data yielded $P \leq 0.05$ for $T = 3$ and 5 min.

Forskolin

The potential involvement of the cAMP-dependent protein kinase A pathway in the 25(OH)D$_3$-mediated efflux of calcium from intestinal cells was investigated. Figure 9 depicts the results of the time course studies with 20 µM forskolin. When expressed relative to controls, $^{45}\text{Ca}$ values for forskolin treated cells were 114%, 107%, 121%, 117%, and 139% of the controls at 1, 3, 5, 7, and 10 min, respectively, after addition of test substances. Statistical analysis of the data revealed a significant increase 10 min after forskolin ($P<0.05$).
Fig. 6. $^{45}$Ca uptake in enterocytes treated with 100 nM 25(OH)D$_3$ in combination with 6.5 nM 24,25(OH)$_2$D$_3$ (closed symbols) or the vehicle ethanol (open symbols, <0.01% v/v, final concentration). Cells were isolated, incubated, sampled and processed as described in Fig. 1. Values represent mean ± SEM for four independent experiments.
Fig. 7(A) Effect of age on 25(OH)D₃-mediated ⁴⁵Ca uptake in enterocytes isolated from (A) 7 wk males; (B) 14 wk males; or (C) 28 wk males. Cells were treated with vehicle (open symbols; <0.01% ethanol v/v, final concentration) or 25(OH)D₃ (closed symbols), incubated and processed as described in the legend to Fig. 2. Values represent mean ± SEM. Significant differences were as described in the legend to Fig. 4.
Fig. 8. $^{45}$Ca uptake in enterocytes treated with a calcium channel activator, 2 µM BAY K 8644 (closed symbols) or the vehicle DMSO (open symbols, <0.2% v/v final concentration). Cells were isolated, incubated, sampled and processed as described in Fig. 1. Values represent mean ± SEM for three independent experiments.

* P<0.05, relative to corresponding control value
Fig. 9. \(^{45}\text{Ca}\) uptake in enterocytes treated with 20 µM forskolin (closed symbols) or the vehicle DMSO (open symbols, <0.2% v/v, final concentration). Cells were isolated, incubated, sampled and processed as described in Fig. 1. Values represent mean ± SEM for six independent experiments.

* P<0.05, relative to corresponding control value
TPA

Time course studies with the protein kinase C activator 12-o-tetradecanoyl-13-phorbol acetate (TPA) were undertaken. Isolated enterocytes labeled with $^{45}$CaCl$_2$ as described above were treated with 100 nM TPA or the vehicle DMSO (<0.2% v/v, final concentration). The results of the experiments are illustrated in Fig. 10. From the figure it is evident that the $^{45}$Ca (treated/average basal) levels in the treated cells are not appreciably different from those of the controls. The $^{45}$Ca treated/average basal levels in the controls were 1.07 ± 0.06, 0.95 ± 0.05, 0.87 ± 0.04, 0.82 ± 0.04 and 0.77 ± 0.04, respectively, for T = 1, 3, 5, 7, and 10 min. In the TPA treated cells, corresponding values were 0.92 ± 0.04, 0.93 ± 0.04, 0.92 ± 0.04, 0.92 ± 0.06, and 0.86 ± 0.05. Statistical analyses for the data yielded P<0.05 for T = 10.

Effects of 25(OH)D$_3$ on Protein Kinases A and C

The potential involvement of protein kinases (both PKA and PKC) in the 25(OH)D$_3$ dependent rapid stimulation of Ca$^{2+}$ handling was investigated. Isolated intestinal cells were treated with 100 nM 25(OH)D$_3$ or the vehicle ethanol (<0.01% v/v final concentration) and harvested at the indicated time points. The cell pellets, collected by centrifugation, were extracted as described in Materials and Methods for protein kinase A or C activities. Results for the PKA assay are shown in Fig. 11. Protein kinase A values in 25(OH)D$_3$-treated cells increased 190% and 178% of corresponding control values at 1 and 3 min. The effect however was not sustained after the 3 min time point.
The PKA activity (percent of total activity) in the controls (mean ± SEM) at T = 1, 3, 5, 7, and 10 min was 15.67 ± 5.75, 18.06 ± 2.28, 21.60 ± 4.82, 30.94 ± 3.74, and 23.39 ± 2.06, respectively (n=3), while in the seco steroid treated cells corresponding values were 29.64 ± 4.38, 32.22 ± 5.82, 23.42 ± 1.94, 31.08 ± 2.52, and 30.33 ± 6.62 (n=3).

Statistical analysis for the time points yielded a significant difference only at T= 1 min (P= 0.013). Figure 12 illustrates the results obtained from the assay for protein kinase C activity. No significant differences in the levels of PKC activity between the controls and the treated cells were observed although a modest 25(OH)D₃-stimulated increase occurred at T=7 min. The PKC activity (percent average basal) in the controls (mean ± SEM) at T =1, 3, 5, 7, and 10 min was 0.94 ± 0.09, 1.06 ± 0.17, 1.06 ± 0.13, 1.05 ± 0.07, and 1.08 ± 0.08, respectively (n=3); while in the treated cells corresponding values were 0.97 ± 0.22, 1.03 ± 0.17, 0.89 ± 0.15, 1.52 ± 0.21, and 1.25 ± 0.15. Statistical analyses for all the time points yielded P>0.05.

Discussion

Early studies on 25(OH)D₃ have suggested that this seco-steroid, which is formed in the liver, is approximately 2-5 times more active than the parent vitamin D molecule in all systems known to be responsive to vitamin D₃ (Blunt et al., 1968; Tanaka and DeLuca, 1973). However, during the same time period it was also reported that 25(OH)D₃ is inactive at physiological concentrations, and that it requires further conversion to an active hormonal form (Boyle et al., 1972; Holick et al., 1972). Thus, the status of 25(OH)D₃ as either an inactive precursor or vitamin D metabolite with its own activity, has not been resolved.
Fig. 10. $^{45}$Ca uptake in enterocytes treated with 100 nM phorbol myristate acetate (a protein kinase C activator; closed symbols) or the vehicle DMSO (open symbols, <0.2% v/v final concentration). Cells were isolated, incubated, sampled and processed as described in Fig. 1. Values represent mean ± SEM for seven independent experiments.

* $P<0.05$
Fig. 11. Time course of 25(OH)D$_3$ effect on protein kinase A activity. Cells were isolated by the citrate chelation protocol. Duplicate 100 µl aliquots were removed in at T= -5 and -1 to establish basal values. Addition of the steroid (closed symbols) or vehicle (ethanol) occurred at T=0. Additional duplicate aliquots were taken at T= 1, 3, 5, 7, and 10 min. Each aliquot was added to 900 µl of GBSS and centrifuged for 10 min at 1000 X g (4°C). The supernatants were decanted, the insides of the tubes swabbed and the harvested cell pellets were homogenized and assayed for protein kinase A activity according to instructions in commercially available kits (Invitrogen-Gibco). Values represent mean ± SEM for three independent experiments.

* P<0.05, relative to corresponding control value
Fig. 12. Time course of 25(OH)D₃ effect on protein kinase C activity. Cells were isolated, incubated, sampled, and processed as described in Fig. 12, and assayed for protein kinase C activity according to instructions in commercially available kits (Invitrogen-Gibco). Values represent mean ± SEM for three independent experiments.
A few earlier studies have indicated that 25(OH)D₃, the precursor to 1,25(OH)₂D₃, may have metabolic effects of its own in regulating cell growth and calcium transport (Olson and DeLuca, 1969; Heaney et al., 1977; Bell et al., 1988; Heaney et al., 1989). Furthermore, Teegarden et al. (2000) have recently reported characterization of a 25-hydroxyvitamin D binding protein from human intestinal cells (Caco-2 cells) which binds to 25(OH)D₃ with at least 1000-fold greater affinity than 1,25(OH)₂D₃ or 24,25(OH)₂D₃. Gacad et al. (1996) have identified a 25(OH)D₃ binding protein, that influenced gene expression (Wu et al., 2000). Recently, Larsson et al. (2002) have demonstrated that 25(OH)D₃ is an active regulator of intestinal Ca²⁺ uptake at physiological concentrations in the Atlantic cod (Gadus morhua).

The present study demonstrates that 25(OH)D₃ is an active metabolite and that its rapid effects on calcium uptake can be studied in isolated chick enterocytes. Using physiological concentrations of the metabolite, 25(OH)D₃ decreases the cellular ⁴⁵Ca levels, relative to controls, within 1-7 min of addition. The decrease in cellular ⁴⁵Ca could be due to either an inhibition of uptake or stimulation of ⁴⁵Ca extrusion relative to the uptake process. Since 25(OH)D₃ has previously been shown to stimulate net calcium transport in the perfused duodenal loop (Nemere et al., 1984, Nemere, 1996a), it is likely that the rapid effects of 25(OH)D₃ in isolated enterocytes represent enhanced calcium extrusion. Larsson et al. (2002) have suggested that in the Atlantic cod (Gadus morhua) 25(OH)D₃ increases the intestinal mucosa-to-serosa transport by increasing Ca²⁺
extrusion through Na+/Ca\(^{2+}\) exchangers across the basal lateral membrane concurrent with an increase in Ca\(^{2+}\) uptake through L-type Ca\(^{2+}\) channels.

Dose response studies indicate an apparent biphasic effect of 25(OH)D\(_3\) in stimulating rapid calcium extrusion from isolated chick intestinal cells, in which stimulation occurs at physiological doses and inhibition at high concentrations. This is characteristic of membrane receptors (de Meyts, 1976). A concentration of 25(OH)D\(_3\) of 25 nM was found to have an insignificant effect on \(^{45}\)Ca levels in the cells, relative to controls while increasing effects are observed at 50-100 nM steroid. Maximal effects were seen at a dose of 100 nM 25(OH)D\(_3\). When the concentration of 25(OH)D\(_3\) is increased to 300 nM an apparent loss of stimulation on \(^{45}\)Ca extrusion occurs.

Moreover, the effect produced by 25(OH)D\(_3\) in isolated intestinal cells was distinct from the effect of 1,25(OH)\(_2\)D\(_3\) on isolated intestinal cells. 1,25(OH)\(_2\)D\(_3\), which has been considered to be the only hormonally active form of vitamin D\(_3\) has little effect on \(^{45}\)Ca handling in chick enterocytes from vitamin D-replete animals (Nemere and Campbell, 2000), although 1,25(OH)\(_2\)D\(_3\) stimulates \(^{45}\)Ca transport in perfused duodenal loops (Nemere et al., 1984; Yoshimoto and Norman, 1986).

The current work also demonstrates that 25(OH)D\(_3\) is capable of rapidly stimulating (within 1-7 min) calcium movement in the intestinal cells of the chicken. It could be possible, keeping in mind the pivotal role of calcium in many physiological processes, that 25(OH)D\(_3\) is an active metabolite of vitamin D\(_3\). 25(OH)D\(_3\) might be useful when an animal reaches a Ca\(^{2+}\) deficient state, and this important ion could be
rapidly obtained from ingested Ca$^{2+}$ without delay due to transcription, or the need for conversion of 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$.

The acute nature of 25(OH)D$_3$ stimulation suggests a signal transduction pathway independent of genomic regulation. Various lines of evidence have indicated that the vitamin D hormone system affects voltage operated calcium channels in the perfused chick intestine (de Boland et al., 1990), heart and muscle (de Boland and Boland, 1987; Selles and Boland, 1991), osteoblasts (Lieberherr, 1987) and pituitary cells (Tornquist and Tashjian, 1989). The ability of 25(OH)D$_3$ to increase the activity of L-type Ca$^{2+}$ channels has been demonstrated in the Atlantic cod (Gadus morhua) enterocytes by Larsson et al. (2002). Caffrey and Farach-Carson (1989), Yukhiro et al., (1994), and Takeuchi and Guggino (1996) using osteoblasts have shown that 25(OH)D$_3$ increases the period during which L-type Ca$^{2+}$ channels stay open, leading to an increased efflux of extracellular Ca$^{2+}$.

Tumor promoting phorbol esters have been shown to substitute for naturally occurring diacylglycerol in stimulating protein kinase C (Kraft and Anderson, 1983). These compounds have been widely used to study PKC-mediated biological responses in a variety of cells (Bell, 1986). Furthermore, increases in the intracellular second messengers (cAMP and protein kinase C) have been postulated to mediate 1,25(OH)$_2$D$_3$ activation of calcium channels (de Boland and Norman, 1990; Fernandez et al., 1990; Selles and Boland, 1991; Massheimer and de Boland, 1992; Massheimer et al., 1994).

Stimulation of adenylate cyclase in the intestinal epithelial cells by 1,25(OH)$_2$D$_3$ and the possible participation of its product, cAMP, in Ca$^{2+}$ transport has been reported
In addition, 1,25(OH)$_2$D$_3$ has been shown to stimulate protein kinase (PKC) dependent substrate phosphorylation activity in the U4937 human monoblastoid cell line (Ways et al., 1987).

However, none of the signal transduction agonists are able to reproduce the 25(OH)D$_3$ induced rapid effects of calcium extrusion from isolated intestinal cells. Forskolin-, BAY K8644-, and phorbol-treated cells have higher levels of $^{45}$Ca (treated/average basal) relative to controls, indicating an increase in uptake of $^{45}$Ca.

Further experiments were carried out to determine PKA and PKC activities in control and 25(OH)D$_3$ treated intestinal cells in order to elucidate whether activation of either one or both protein kinases exhibited involvement in the rapid effects on calcium extrusion. The data indicate that 25(OH)D$_3$ does not elevate the levels of PKC significantly, but that PKA activity is stimulated to significant levels within one minute, and for a brief period of time. Thus it is possible to suggest that protein kinase A activation is linked to 25(OH)D$_3$ mediated $^{45}$Ca extrusion.

However, since forskolin treatment results in uptake of $^{45}$Ca in the cells rather than enhanced extrusion, it is likely that the results with forskolin represent a non-specific increase in PKA enzyme activity. PKA activated by 25(OH)D$_3$ may, through a more localized action, signal other pathways that enhance extrusion. The current work also demonstrates that physiological levels of 24,25(OH)$_2$D$_3$ inhibit 25(OH)D$_3$ enhanced calcium extrusion in isolated intestinal cells. Antagonism of the stimulatory effect was evident at 1 minute after 24,25(OH)$_2$D$_3$ addition. This is in line with the findings of Larsson et al. (2002) that 24R,25(OH)$_2$D$_3$ and 25(OH)D$_3$ have rapid antagonistic effects
on Ca$^{2+}$ homeostasis in Atlantic cod enterocytes. 24,25(OH)$_2$D$_3$ has been reported to suppress the rapid action of 1,25(OH)$_2$D$_3$ on calcium transport in the perfused duodenal loop system (Nemere, 1999). 24,25(OH)$_2$D$_3$ also suppressed 1,25(OH)$_2$D$_3$ enhanced calcium uptake in osteoblasts (Khoury et al., 1995). Although it might be tempting to speculate that 24,25(OH)$_2$D$_3$ inhibits the action of 25(OH)D$_3$ by binding to and blocking the 25(OH)D$_3$ receptor, reports indicate that the two metabolites may have distinctly different receptors (Teegarden et al., 2000; Nemere et al., 2002). In addition, a recent study on the newly identified, non nuclear binding activity for 24,25(OH)$_2$D$_3$ indicates that it has a low affinity for 25(OH)D$_3$ (Nemere et al., 2002). The data therefore suggest that 24,25(OH)$_2$D$_3$ appears to be an endogenous antagonist of both 25(OH)D$_3$ and 1,25(OH)$_2$D$_3$ with reference to calcium transport in the intestinal cells.

Studies in fish such as the European eel (Chartier et al., 1979) and American eel (Anguilla rostrata; Fenwick et al., 1984) have shown that vitamin D$_3$, the parent vitamin D metabolite, is equipotent to 1,25(OH)$_2$D$_3$ in stimulating intestinal Ca$^{2+}$ uptake. These findings indicate that the calcitropic effects of the vitamin D endocrine system may have changed from less polar metabolites like vitamin D$_3$ and 25(OH)D$_3$ to more polar metabolites like 1,25(OH)$_2$D$_3$ during vertebrate evolution.

It is a well-known fact that there is a decline in calcium absorption and increased loss of bone with advancing age. Studying the effect of age on the response to 25(OH)D$_3$ by isolated chick enterocytes was one of the objectives of this study.

Results demonstrate that the magnitude of the response to 25(OH)D$_3$ decreases with age. This finding is similar to reports of others (Armbrecht et al., 1980; Liang et al.,
1990) on the decrease in effectiveness of vitamin D metabolites with regard to intestinal calcium transport. The combined data suggest that chick intestinal cells have decreased sensitivity to all stimulatory metabolites of vitamin D with increasing age. The decrease in the responses could be due to a reduction in certain components, such as receptors or signal transduction pathways that are necessary for biological activity as a result of exposure to steroid.

The overall results of the present study indicate that 25(OH)D₃ may have a physiologically significant action in isolated intestinal cells, and that this action can be suppressed by 24,25(OH)₂D₃. Further research will determine the actual signal transduction pathways in response to 25(OH)D₃ exposure, the identification and characterization of the 25(OH)D₃ receptor in chick intestinal cells, the mechanism of inhibition of 25(OH)D₃ action by 24,25(OH)₂D₃ action, and the reasons for a decrease in magnitude of response to 25(OH)D₃ with age. Finally, in order for 25(OH)D₃ to be considered hormonally active, definitive experiments must be designed to test its effects independent of 1,25(OH)₂D₃ in vivo.
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