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$1,25(OH)_2D_3$-Mediated Phosphate Uptake in Isolated Chick Intestinal Cells: Effect of $24,25(OH)_2D_3$, Signal Transduction Activators, and Age

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Abstract We have previously reported an absence of a $1,25(OH)_2D_3$-mediated effect on ^{45}Ca handling by intestinal epithelial cells isolated from normal chicks (Nemere and Campbell [2000] Steroids 65:451–457). In the current work, we provide evidence that in similar cell preparations, $1,25(OH)_2D_3$ increased ^{32}P uptake within 5 min of addition, and reached 150% of controls after 10 min ($P < 0.05$). Both isolated enterocytes and the perfused duodenal loop system exhibited apparent biphasic dose–response curves for $1,25(OH)_2D_3$-stimulated ^{32}P uptake and transport, and inhibition of stimulation by $24,25(OH)_2D_3$. A comparison of signal transduction activators demonstrated the following parallels in both isolated intestinal cells and perfused duodena: lack of effect of forskolin (a protein kinase (PK) A activator) on ^{32}P handling, but simulation by BAY K8644 (a calcium channel activator) and phorbol ester (a PKC activator). Finally, we tested the effect of $1,25(OH)_2D_3$ on phosphate uptake in epithelial cells isolated from birds of increasing ages (7, 14, and 28 wk). In contrast to the robust response of cells from young, growing chicks, $1,25(OH)_2D_3$ had no effect on enterocytes from 14 or 28 wk birds. Western analyses with Ab 099 against the $1,25(OH)_2D_3$ ($1,25D_3$)-Membrane-Associated Rapid Response Steroid (MARRS) binding protein revealed a decrease in average density of the immunoreactive band with age. PKC activity determined in isolated epithelial cells exhibited a decrease in average basal (control) activity with age, as well as a decrease in response to $1,25(OH)_2D_3$ activation. In enterocytes from 7–14- or 28-week birds, PKC was enhanced 170, 120, and 105% of controls, respectively. The combined data validate ^{32}P uptake in isolated enterocytes as a model system to study $1,25D_3$-MARRS protein function, and indicate that for phosphate transport, the rapid actions of $1,25(OH)_2D_3$ are physiologically more important in growing animals than immature ones. J. Cell. Biochem. 86: 497–508, 2002.

Key words: enterocytes; signal transduction; $1,25D_3$-MARRS binding protein; rapid actions

The rapid effects of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] have been described in many isolated cell systems [Nemere and Farach-Carson, 1998; Pietras et al., 2001]. In isolated intestinal epithelial cells of normal, vitamin D-replete rats, $1,25(OH)_2D_3$ increases ^{45}Ca uptake [Nemere and Szego, 1981a,b], as well as ^{32}P uptake [Karsenty et al., 1985]. However, enterocytes isolated from normal chicks fail to exhibit a response to $1,25(OH)_2D_3$ in vitro with respect to ^{45}Ca uptake [Nemere and Campbell, 2000]. In this study, we tested isolated chick intestinal epithelial cells for their ability to respond to $1,25(OH)_2D_3$ with altered ^{32}P handling. The isolated enterocyte model system was validated relative to the perfused duodenal loop system of normal chicks through comparison of dose–response curves for $1,25(OH)_2D_3$, and effects of $24,25(OH)_2D_3$ or signal transduction activators. We have postulated that the rapid effects of $1,25(OH)_2D_3$ are mediated by a $1,25D_3$-membrane-associated rapid response steroid (MARRS) binding protein, located at the basal lateral membrane; the protein has previously been isolated [Nemere et al., 1994], and an antibody (Ab 099) generated toward the N-terminal

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sequence [Nemere et al., 2000]. The results indicate that the cell model system will be suitable for testing the effect of $1,25D_3$-MARRS-binding protein [Nemere et al., 2001] ablation with respect to transmission of ligand-induced signaling.

MATERIALS AND METHODS

Animals and Surgical Procedures

White leghorn cockerels were obtained on the day of hatch and generally raised for 4–8 weeks, on a commercially available vitamin D-replete diet before experimentation [Nemere, 1996a]. All procedures were approved by the Institutional Animal Use and Care Committee at Utah State University.

Cell Isolation

On the day of use, chicks were anesthetized with chloropent (0.3 ml/100 g body weight), and the duodenal loop was surgically removed to ice-cold saline. After chilling for 15 min, the pancreas was excised and the duodenal loop everted and rinsed in ice-cold saline. Cells were isolated as previously described [Nemere and Campbell, 2000]. The loop was transferred to a plastic beaker containing 30 ml of citrate chelation solution (96 mM NaCl, 27 mM $NaCitrate \cdot 2H_2O$, 1.5 mM KCl, 8 mM KH_2PO_4, 5.6 mM Na_2HPO_4, pH 7.0) at 23°C, gently stirred for 15 min, and then transferred to fresh chelation solution. The isolated cells in suspension were transferred to two 50-ml polypropylene centrifuge tubes on ice. The isolation procedure was performed a total of three times. Cells were collected by low speed centrifugation (500*g*, 5 min, 4°C). The supernatant was decanted and while still inverted, the inside of the tube was swabbed with a Kimwipe. Cell pellets were resuspended in Gey's Balanced Salt Solution (GBSS, 119 mM NaCl, 4.96 mM KCl, 0.22 mM KH_2PO_4, 0.89 mM Na_2HPO_4, 1.03 mM $MgCl_2 \cdot 6H_2O$, 0.28 mM $MgSO_4 \cdot 7H_2O$, 0.9 mM $CaCl_2 \cdot 2H_2O$) buffered to pH 7.4, and containing 0.1% bovine serum albumin (RIA grade, Sigma Chemical Co., St. Louis, MO) as follows: the pellet was gently dispersed along the inside walls of the tube with a Teflon-coated rod. Resuspension medium (10 ml per pellet) was added a drop at a time until a fairly liquid suspension was achieved and then medium was added more rapidly. This procedure minimized cell clumping. A 5-ml aliquot of cell suspension was then combined with 2 μCi/ml of $H_3{}^{32}PO_4$ (Perkin Elmer New England Nuclear, Boston, MA).

Time Course and Dose–Response Studies

The suspension with label was divided into two groups (2 ml each), and duplicate 100 μl aliquots from each group removed at $T = -5$ and -1 min. Each aliquot was pipetted into 900 μl of ice-cold GBSS, a procedure that served to stop further uptake and wash the cells in buffer containing excess unlabeled phosphate. At $T = 0$, one suspension was treated with vehicle (0.012% ethanol, final concentration) and the other with $1,25(OH)_2D_3$. At $T = 1, 3, 5, 7$, and 10 min, duplicate aliquots were removed to 900 μl of ice-cold GBSS, as described above. Cell viability (98% by Trypan blue exclusion [Nemere et al., 2000]) remains undiminished under these conditions. The early rather than extended time points were chosen for analyses to allow comparison with signal transduction activators (see below).

The samples were then centrifuged (1,000*g*, 10 min), the supernatants decanted, and the inside of the tubes swabbed with a Kimwipe, while the tubes were still in the inverted position. The pellets were processed as described below for protein and radioactivity. For dose response studies, 10, 50, 130, or 300 pM $1,25(OH)_2D_3$ was tested. For experiments, testing the effect of $24,25(OH)_2D_3$ in combination with $1,25(OH)_2D_3$, the protocols described above were used except that cells were treated with vehicle (0.012% ethanol, final concentration) or a combination of 130 pM $1,25(OH)_2D_3$ and 6.5 nM $24,25(OH)_2D_3$ at $T = 0$.

Effect of Age on $1,25(OH)_2D_3$-Stimulated ^{32}P Uptake by Isolated Intestinal Cells

Cells were isolated from male chickens that were 7, 14, or 28 week of age. These ages cover growth (a period of rapid bone formation) and adulthood when the demand for bone mineralization decreases. Enterocytes from each age group were incubated as described for time course studies (see above) with either vehicle or 130 pM $1,25(OH)_2D_3$.

Effect of Signal Transduction Activators in Isolated Intestinal Cells

Isolated enterocytes were incubated in a manner similar to that described for the time course studies, but treated with either vehicle

(0.2% DMSO, final concentration), 10 μM forskolin (a PKA activator [Seamon and Daly, 1981]), 100 nM phorbol 12-myristate 13-acetate (a protein kinase C (PKC) activator [Kraft and Anderson, 1983]), or 2 μM BAY K 8644 (a calcium channel activator; all from Sigma) added at T = 0.

Perfusion of Duodenal Loops

Procedures for perfusion studies were as described before [Nemere, 1996a]. Briefly, duodena were perfused through the celiac artery with control medium: GBSS containing $NaHCO_3$, and aerated with 95% O_2/5% CO_2, was delivered by the main pump and combined with media containing 1.25 mg/ml BSA and 0.05 μl/ml ethanol from a side pump for a 20-min basal period, beginning with the initiation of lumenal perfusion with GBSS (containing 2 μCi/ml $H^{32}PO_4$). Samples were collected at 2-min intervals during the last 10 min to determine average basal transport rates. Vascular perfusion was then continued either with control medium, or test substances, as indicated in the figures, for an additional 40 min, and aliquots of the venous effluent taken at 2-min intervals for determination of ^{32}P.

Quantitation of Results

The harvested cell pellets were resuspended in reagent grade water and analyzed for protein using the Bradford reagent (Bio-Rad, Hercules, CA) with bovine gamma globulin as standard (Sigma), and radioactivity by liquid scintillation spectrophotometry. Radionuclide (CPM) was related to cellular protein in each aliquot. After normalizing values to average basal cpm/mg protein, the specific activity (cpm/mg protein) in each sample at a given time point after addition of test substance was then expressed as percent of control.

Determination of PKC Activity

Time course studies with isolated intestinal cells indicated that optimal responsiveness to $1,25(OH)_2D_3$ occurred within 5 min [Nemere, 1999]. Approximately 10^6 cells were treated with appropriate vehicle, 130 pM $1,25(OH)_2D_3$, or 2 μM BAY K8644 for 5 min and then pelleted at low speed. For experiments with age groups, the pellets were frozen at −20°C until all samples were collected, and then processed further on the same day. Pellets were homogenized in 20 mM Tris, pH 7.5, containing 0.5 mM EDTA, 0.5 mM EGTA, 0.5% Triton X-100, and 25 μg/ml aprotinin and leupeptin. The high-speed supernatants (14,000*g*, 2 min, 4°C) were diluted 1:4 with buffer that did not contain detergent, and analyzed for proteins. After adjusting protein levels to 1 μg/μl, 10 μl were taken for analysis of enzyme activity, according to instructions supplied with the kit (Invitrogen GIBCO Life Technologies, Waverly, MA). Aliquots were analyzed for the ability to phosphorylate exogenous substrate (50 μM myelin basic protein) in the presence or absence of a specific PKC inhibitor, corresponding to the psuedosubstrate region PKC (19–36). All samples were incubated at 23°C for 20 min to allow binding of inhibitor before the addition of buffer containing [^{32}P] ATP-20 μM ATP and acetylated myelin basic protein. After an additional 5-min incubation at 30°C, 25-μl samples were spotted onto phosphocellulose disks. The disks were then washed two times in 1% phosphoric acid and two times in water before determination of retained radioactivity by liquid scintillation counting. Values were expressed as (total activity)—(inhibitor sensitive activity) and related to extract protein.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS–PAGE) and Western Analysis

Basal lateral membranes (BLM) were prepared from 7-, 14-, and 28-week-aged birds by a combination of differential and Percoll gradient sedimentation [Nemere, 1996b] and stored at −20°C for subsequent analyses of the $1,25D_3$-MARRS binding protein. Solubilized membranes (15 μg protein per well) from each of the three age groups (n = 3) were separated on the same 13-cm resolving slab gels. Colored molecular weight standards (Bio-Rad) were run simultaneously to monitor the subsequent transfer of protein to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA) by electroblotting in a Bio-Rad Semi-dry transfer system (15 V, 25 min, 23°C). Western analyses were performed according to Millipore protocols and as described elsewhere [Nemere et al., 2000]. The membrane was incubated for 1 h at 37°C in blocking buffer (150 mM NaCl, 10 mM Na_2HPO_4, pH 7.4, with 0.5% w/v nonfat dry milk powder). The membrane was then washed three times, 5 min each, in washing solution (0.1% BSA in Tris-buffered saline (TBS), pH 7.4: 150 mM NaCl, 20 mM Tris,

0.02% NaN_3), before an overnight exposure (4°C) to primary antibody. Ab 099, directed against the N-terminal sequence of the 1,25D_3-MARRS binding protein [Nemere et al., 2000] was used at a 1:5,000 dilution in antibody incubation solution (1% w/v BSA, 0.05% v/v Tween-20 in TBS). The following morning, the membrane was again washed 3 min × 5 min, incubated for 2 h (23°C) with alkaline phosphatase-conjugated secondary antibody (Sigma) diluted at 1:15,000. Three additional washes were performed prior to the colorimetric reaction. For visualization, the membrane was incubated with chromogenic substrate (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium, both from Sigma) in 10 mM Tris. For quantitation, Western blots were scanned into a computer file and densitometrically analyzed.

Statistical Analyses

Statistical comparisons between two treatment groups were made by Student's *t*-test for paired observations. Results of dose–response analyses were compared by ANOVA. All values are presented as mean ± SEM. Statistical significance was set at $P \leq 0.05$.

RESULTS

Effects of 1,25$(OH)_2D_3$ on Phosphate Uptake in Isolated Cells

Time course studies on ^{32}P uptake in isolated cells were undertaken with a range of 1,25$(OH)_2D_3$ concentrations (Fig. 1). Basal values for treated and control cells were generally 16,000 cpm/mg protein. In order to compare results, the average basal values (cpm/mg protein) were used to divide cpm/mg protein obtained at each time point. Figure 1A depicts a representative graph using such values. In subsequent figures, results are expressed as percent of control for easier comparison between doses. Treatment of cells with 10 pM 1,25$(OH)_2D_3$ failed to alter ^{32}P levels from those observed in controls (Fig. 1B). Figures 1C,D depict results obtained from isolated intestinal cells treated with either vehicle (○), 50 pM 1,25$(OH)_2D_3$ (Fig. 1C, ●) or 130 pM 1,25$(OH)_2D_3$ (Fig. 1D, ●). Both 50 pM 1,25$(OH)_2D_3$ and 130 pM 1,25$(OH)_2D_3$ promoted an early rise in the uptake of ^{32}P, increasing to ~130 and 151%, respectively, of corresponding control values at 10 min ($P < 0.05$). Figure 1E illustrates the time course for phosphate uptake in response to 300 pM 1,25$(OH)_2D_3$. An early rise in the phosphate uptake was also stimulated by 300 pM 1,25$(OH)_2D_3$, but to a lesser extent than the lower concentrations. Uptake of ^{32}P increased ~123% of the control value at 10 min ($P < 0.05$). Figure 2 summarizes the results of a range of 1,25$(OH)_2D_3$ concentrations on phosphate uptake at 10 min. This comparison suggested a biphasic dose–response curve: an absence of stimulated ^{32}P uptake was evident at 10 pM hormone, moderate stimulation was found at 50 pM steroid, a plateau of optimal response was obtained at 130 pM 1,25$(OH)_2D_3$, and a slight suppression at 300 pM seco-steroid. On the basis of these results, the optimal concentration of hormone, 130 pM 1,25$(OH)_2D_3$, was used in subsequent experiments with isolated cells.

Effect of 24,25$(OH)_2D_3$ With 1,25$(OH)_2D_3$ on Phosphate Uptake in Isolated Cells

In previous studies, 6.5 nM 24,25$(OH)_2D_3$ failed to stimulate phosphate transport and attenuated the stimulatory effect of 1,25$(OH)_2D_3$. Thus, experiments were done to determine the interaction of 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ on phosphate uptake in isolated chick intestinal cells. Figure 3 depicts the results of isolated intestinal cells treated with vehicle (0.012% ethanol, final concentration) or a combination of 130 pM 1,25$(OH)_2D_3$ and 6.5 nM 24,25$(OH)_2D_3$. The absence of a difference between controls and steroid-treated suspensions suggested that 24,25$(OH)_2D_3$ apparently exerts an inhibitory effect on 1,25$(OH)_2D_3$-enhanced phosphate uptake in isolated cells (Fig. 3).

Dose–response studies on the effect of 1,25$(OH)_2D_3$ on phosphate transport in the perfused duodenal loop, and attenuation by 24,25$(OH)_2D_3$. 24,25$(OH)_2D_3$ and 1,25$(OH)_2D_3$ were also tested for their action on phosphate transport in perfused duodena. Figure 4 depicts the results of dose–response experiments in which duodena were perfused with control medium (n = 12), 65 pM 1,25$(OH)_2D_3$ (n = 4), 130 pM 1,25$(OH)_2D_3$ (n = 3), 300 pM 1,25$(OH)_2D_3$ (n = 2), 650 pM 1,25$(OH)_2D_3$ (n = 4), and each concentration of 1,25$(OH)_2D_3$ in combination with 6.5 nM 24,25$(OH)_2D_3$ (n = 4, 3, 2, and 4, respectively). Comparison of the 40-min time points indicated that the ratio of treated/average basal (mean ± SEM) was 1.00 ± 0.09 for controls, 1.36 ± 0.13

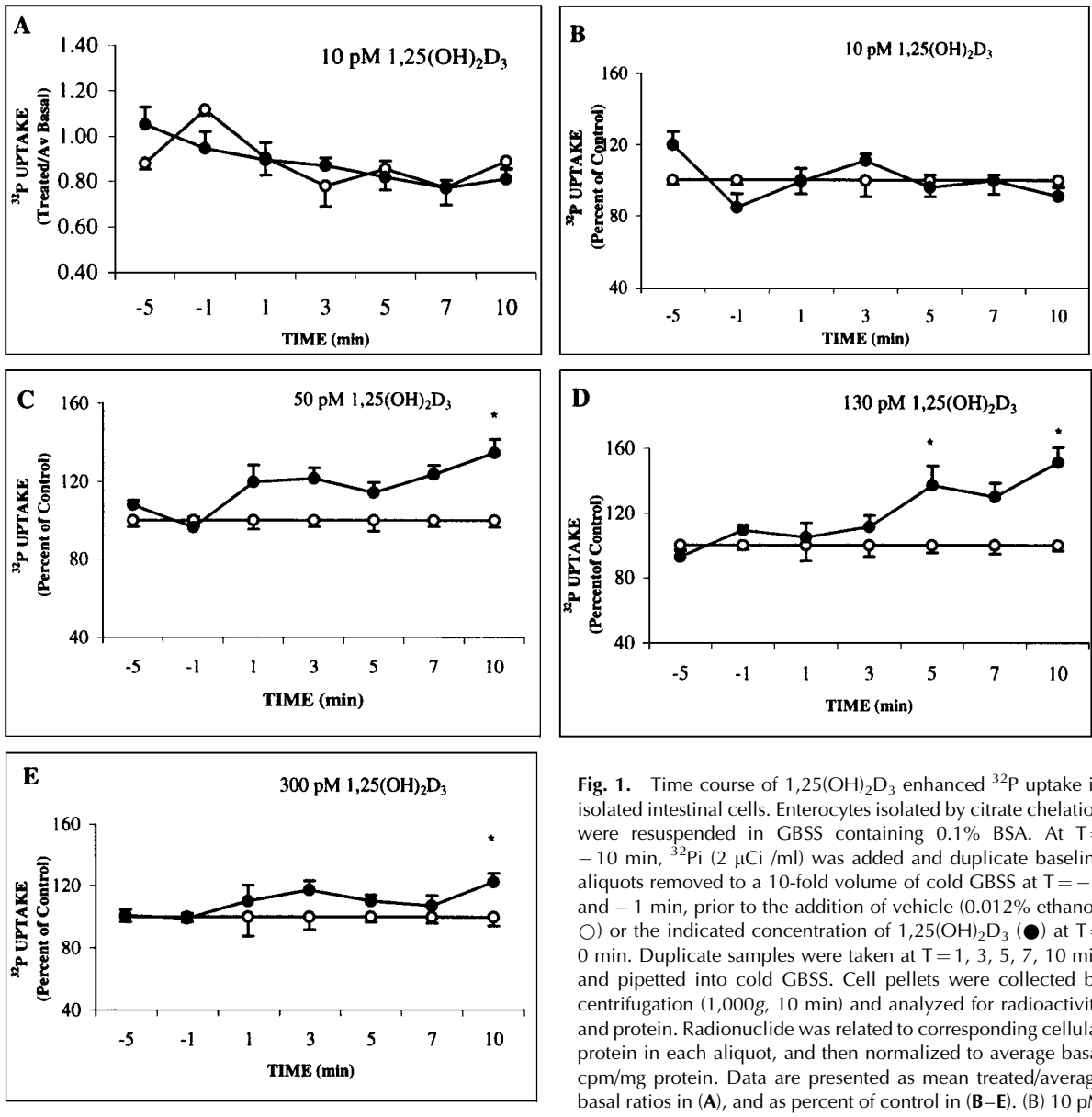

Fig. 1. Time course of 1,25(OH)$_2$D$_3$ enhanced ^{32}P uptake in isolated intestinal cells. Enterocytes isolated by citrate chelation were resuspended in GBSS containing 0.1% BSA. At T = −10 min, ^{32}Pi (2 μCi /ml) was added and duplicate baseline aliquots removed to a 10-fold volume of cold GBSS at T = −5 and −1 min, prior to the addition of vehicle (0.012% ethanol, ○) or the indicated concentration of 1,25(OH)$_2$D$_3$ (●) at T = 0 min. Duplicate samples were taken at T = 1, 3, 5, 7, 10 min and pipetted into cold GBSS. Cell pellets were collected by centrifugation (1,000*g*, 10 min) and analyzed for radioactivity and protein. Radionuclide was related to corresponding cellular protein in each aliquot, and then normalized to average basal cpm/mg protein. Data are presented as mean treated/average basal ratios in (**A**), and as percent of control in (**B**–**E**). (B) 10 pM 1,25(OH)$_2$D$_3$ (n = 3); (C) 50 pM 1,25(OH)$_2$D$_3$ (n = 3); (D) 130 pM 1,25(OH)$_2$D$_3$ (n = 5); (E) 300 pM 1,25(OH)$_2$D$_3$ (n = 5). *$P \leq 0.05$, relative to corresponding control values.

for 65 pM 1,25(OH)$_2$D$_3$, and 1.08 ± 0.04 for 65 pM 1,25(OH)$_2$D$_3$ plus 6.5 nM 24,25(OH)$_2$D$_3$. Values of 1.45 ± 0.05 and 1.15 ± 0.02 were obtained for 130 pM 1,25(OH)$_2$D$_3$ alone and in combination with 24,25(OH)$_2$D$_3$, respectively. For 300 pM 1,25(OH)$_2$D$_3$ alone or in the presence of 24,25(OH)$_2$D$_3$, the ratios were 2.15 ± 0.21 and 0.92 ± 0.12. These results indicated that 24, 25(OH)$_2$D$_3$ significantly decreased the stimulation of phosphate transport by 1,25(OH)$_2$-D$_3$ ($P < 0.05$ for all three different concentrations of 1,25(OH)$_2$D$_3$). Stimulated phosphate transport at 650 pM 1,25(OH)$_2$D$_3$ with or without 6.5 nM 24,25(OH)$_2$D$_3$ was not evident (Treated/AV basal ratios at T = 40 were 1.10 ± 0.10 and 1.01 ± 0.03, respectively). These results were in agreement with the effect of 24,25(OH)$_2$D$_3$ with 1,25(OH)$_2$D$_3$ on phosphate uptake in isolated intestinal cells, in that both model systems exhibit apparent biphasic dose–response curve for 1,25(OH)$_2$D$_3$, although with different maxima, and attenuation of stimulated uptake or transport by 24,-25(OH)$_2$D$_3$.

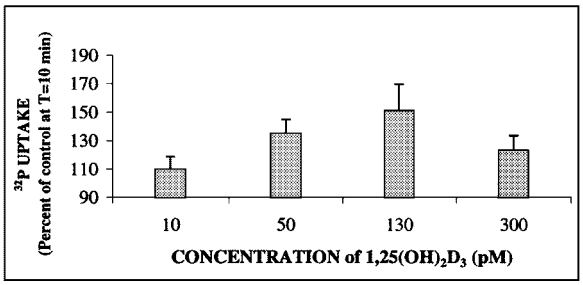

Fig. 2. Summary of time course and dose response studies at T = 10 min. Values represent mean ± SEM.

Effect of Signal Transduction Activators

An additional series of experiments was undertaken with forskolin, phorbol ester, and Bay K 8644. Figure 5A reveals that 10 μM forskolin failed to enhance phosphate uptake in isolated intestinal cells ($P > 0.05$, treated relative to controls for all time points tested). Figures 5B,C illustrate the effects of Bay K 8644 and phorbol ester, respectively, on phosphate uptake in isolated intestinal cells. Enhanced ^{32}P levels relative to controls were found for BAY K 8644 as early as 5 min after addition, achieving 134% of controls ($P < 0.05$), and reaching an apparent maximum of 150% of controls at 7 min ($P < 0.05$; Fig. 5B). Phorbol ester treatment resulted in an apparent increase in phosphate uptake 1 min after addition, to ~125% of controls, a value that was maintained at T = 5–10 min (all, $P < 0.05$). In an attempt to determine whether calcium signaling and PKC were related, isolated intestinal cells were incubated for 5 min with vehicle, 130 pM 1,25(OH)$_2$D$_3$, or 2 μM BAY K8644. Cells were collected by centrifugation and the pellets extracted for determination of PKC activity. As shown in Figure 6, calcium channel activation did not appreciably enhance PKC activity, while the seco-steroid hormone elicited a twofold increase.

Perfusion Studies With Signal Transduction Activators

The signal transduction activators forskolin, phorbol ester, and Bay K 8644 were also tested on phosphate transport in perfused duodena. Figure 7 illustrates the results of duodena perfused with control medium (n = 7), 10 μM forskolin (n = 2), 2 μM BAY K 8644 (n = 2), or 100 nM phorbol myristate acetate (n = 3). At the end of the 40-min treatment period, the ratio of treated/average basal (mean ± SEM) was 1.12 ± 0.21 for controls, 1.18 ± 0.12 for forskolin, 5.15 ± 1.27 for Bay K 8644, and 3.47 ± 1.21 for phorbol ester. Bay K 8644-augmented phosphate transport was statistically significant between 4 and 40 min of perfusion during the treated phase ($P < 0.05$, relative to corresponding controls). Phorbol ester also stimulated phosphate transport, but to a lesser extent than Bay K 8644 with a marginally significant difference compared to control media ($P \approx 0.05$). However, perfusion with forskolin failed to stimulate phosphate transport. Again, these results parallel and validate the isolated intestinal cell model system.

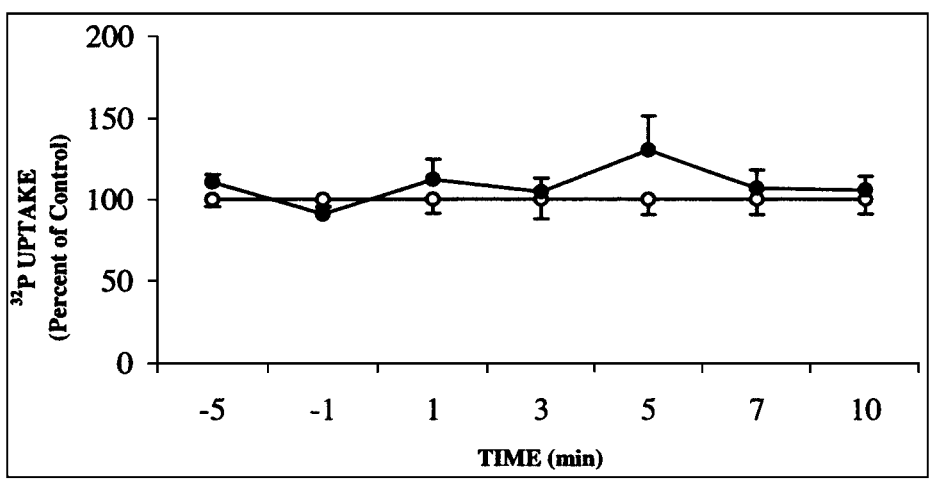

Fig. 3. Effect of 6.5 nM 24,25(OH)$_2$D$_3$ and 130 pM 1,25(OH)$_2$D$_3$ on phosphate uptake in isolated intestinal cells. Conditions were as described in Figure 1. Cells were treated with vehicle (○) or both steroids (●) at T = 0 min. Values represent means ± SEM for six independent experiments.

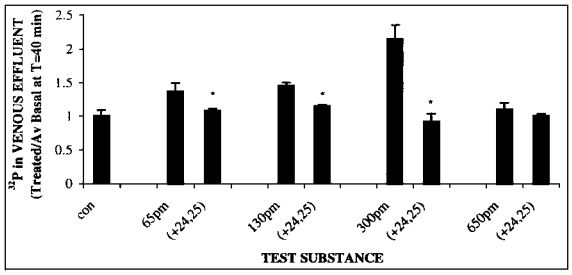

Fig. 4. Effect of increasing concentrations of $1,25(OH)_2D_3$ alone or in combination with $24,25(OH)_2D_3$ on phosphate transport in perfused duodena. Perfusion conditions were as described in text using the indicated concentrations of secosteroid. Data are presented for the 40-min time point of the treated phase. Values represent mean ± SEM. $^*P \leq 0.05$, relative to corresponding values from experiments with $1,25(OH)_2D_3$ alone.

Effect of Age on $1,25(OH)_2D_3$-Stimulated ^{32}P Uptake by Isolated Intestinal Cells

Since decreased intestinal mineral absorption with aging is known to occur in mammalian species [Armbrecht, 1990], another objective of this research was to determine whether the rapid effects of $1,25(OH)_2D_3$ decrease with maturation. Figures 8A,B depict the results of $1,25(OH)_2D_3$-mediated phosphate uptake in isolated cells prepared from duodena of 14- or 28-week birds. Neither the enterocytes from 14- (average body weight 0.92 kg) or 28-week birds (average body weight 1.12 kg) showed an increase in ^{32}P uptake as a result of treatment with 130 pM $1,25(OH)_2D_3$. These results are in marked contrast to those observed with enterocytes prepared from 7-week birds (average body weight, 0.4 kg) (Fig. 1D).

Effect of Age on the $1,25D_3$-MARRS Binding Protein as Judged by Western Analyses

The above study on age and $1,25(OH)_2D_3$-stimulated ^{32}P uptake by isolated intestinal cells indicated that rapid effects of $1,25(OH)_2D_3$ decreased with maturation. One explanation is that the expression of a MARRS binding protein also decreased with aging. To address this question, studies on the $1,25D_3$-MARRS receptor antigen were undertaken by Western analyses in birds of different age groups. The polyclonal antibody (Ab 099) that was used to test for immunoreactivity has been well characterized in chicken intestine [Nemere et al., 2000] and found to be highly specific. For three independent preparations of BLM, 15 μg of protein from 7-, 14-, and 28-week-aged birds were loaded in individual lanes. Figure 9 shows the results of densitometric analyses of the Western blots. The relative band density for

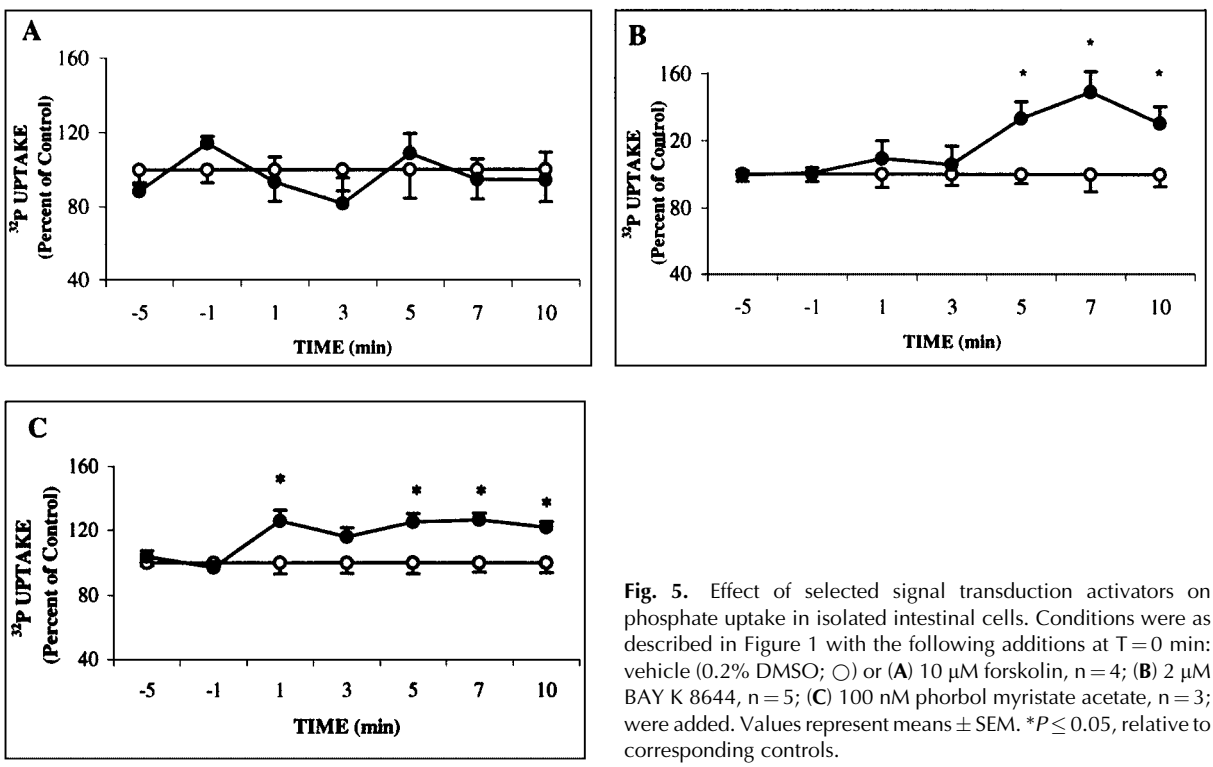

Fig. 5. Effect of selected signal transduction activators on phosphate uptake in isolated intestinal cells. Conditions were as described in Figure 1 with the following additions at T = 0 min: vehicle (0.2% DMSO; ○) or (**A**) 10 μM forskolin, n = 4; (**B**) 2 μM BAY K 8644, n = 5; (**C**) 100 nM phorbol myristate acetate, n = 3; were added. Values represent means ± SEM. $^*P \leq 0.05$, relative to corresponding controls.

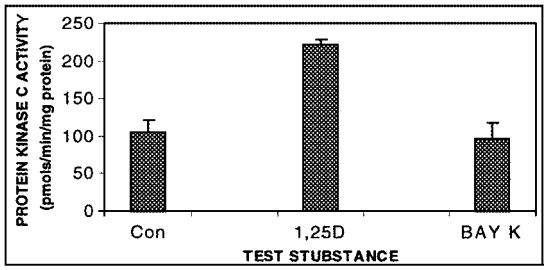

Fig. 6. BAY K8644 does not stimulate PKC activity. Isolated intestinal cells (10^6 per incubation) were treated with vehicle, 130 pM $1,25(OH)_2D_3$, or 2 μM BAY K8644 for 5 min prior to collection of cell pellets by centrifugation. Pellets were extracted and tested for PKC activity as described in the text. Values represent average ± range for duplicate experiments.

immunoreactivity in $1,25D_3$-MARRS binding protein from 7-, 14-, and 28-week-aged birds was 260.3 ± 19.2, 239.7 ± 15.8, and 235.3 ± 2.4 (mean ± SEM), respectively. Although the values are not significantly different, there was a decrease of average $1,25D_3$-MARRS binding protein band density with increasing ages, that paralleled the decrease in $1,25(OH)_2D_3$-stimulated phosphate uptake in isolated intestinal cells.

Effect of $1,25(OH)_2D_3$ on PKC Activity as a Function of Age

Using the 5-min time point, PKC activity in intestinal cells was tested in the different age groups of birds in response to vehicle (basal or control values) or $1,25(OH)_2D_3$. As revealed in Figure 10A, intestinal cells from 7-week-old chicks incubated with control medium in the absence of hormone exhibited a low level of activity (~100 pmols/min/mg protein). When determined within the same assay for enzyme activity, an age-related decrease in average basal PKC activity was observed in enterocytes from 14-week birds (78.78 ± 11.46 pmols/min/mg protein, Fig. 10B) and 28-week birds (58.04 ± 6.59 pmols/min/mg protein, Fig. 10C). In enterocytes from 7-week birds (Fig. 10A), addition of 130 pM $1,25(OH)_2D_3$ resulted in an increase in PKC activity to 174% of controls ($P = 0.05$). The average values for specific activity (pmol/min/mg protein) ± SEM were 95.78 ± 17.84 for controls, and 166.63 ± 36.22 for $1,25(OH)_2D_3$-treated cells (Fig. 10A). In 14-week-old chicks (Fig. 10B), $1,25(OH)_2D_3$ still exerted a stimulatory effect on PKC activity, but at a modest level, increasing activity only to 122% of control groups ($P \approx 0.05$). In preparations from 28-week birds (Fig. 10C), the difference between treated and control groups was no longer significant. These experiments showed that there was a decrease of hormone-stimulated PKC activity in birds of increasing ages, which was paralleled by the age-related decrease of steroid-enhanced ^{32}P uptake in isolated intestinal cells, and decreasing levels of the expression of the $1,25D_3$-MARRS binding protein (Fig. 10).

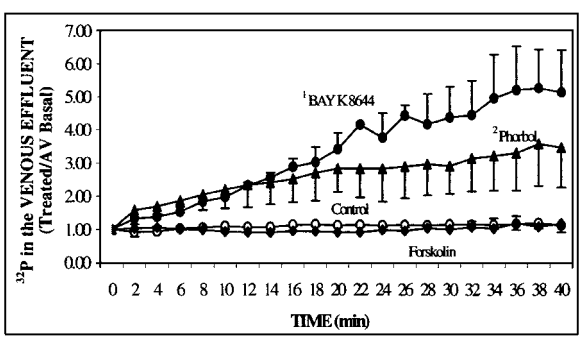

Fig. 7. Comparison of the effect of selected signal transduction activators on phosphate transport in perfused duodena. Perfusion conditions were as described in the text. Values represent mean ± SEM. [1]$P < 0.05$, relative to corresponding controls from 4–40 min; [2]$P \approx 0.05$, relative to corresponding controls from 4–40 min.

DISCUSSION

The work described demonstrates that $1,25(OH)_2D_3$ is capable of directly and rapidly (5–10 min) stimulating phosphate uptake in isolated intestinal cells of normal chicks, an occurrence that is paralleled in many instances by results observed in the perfused duodenal loop. In contrast, while $1,25(OH)_2D_3$ stimulates calcium transport in the perfused duodenal loop [Nemere, 1999], no effect is observed with intestinal epithelial cells isolated from normal chicks [Nemere and Campbell, 2000].

In the present studies, both phosphate uptake in isolated intestinal cells and phosphate transport in perfused duodena were stimulated by physiological concentrations of $1,25(OH)_2D_3$, with apparent suppression of stimulation at higher concentrations of steroid. The optimal concentration of hormone was higher in the perfused duodenal loop than in isolated cells, perhaps because the perfusate is presented to additional tissue prior to reaching the basal lateral membrane of the mucosa. The apparent biphasic nature of the dose–response curve is a

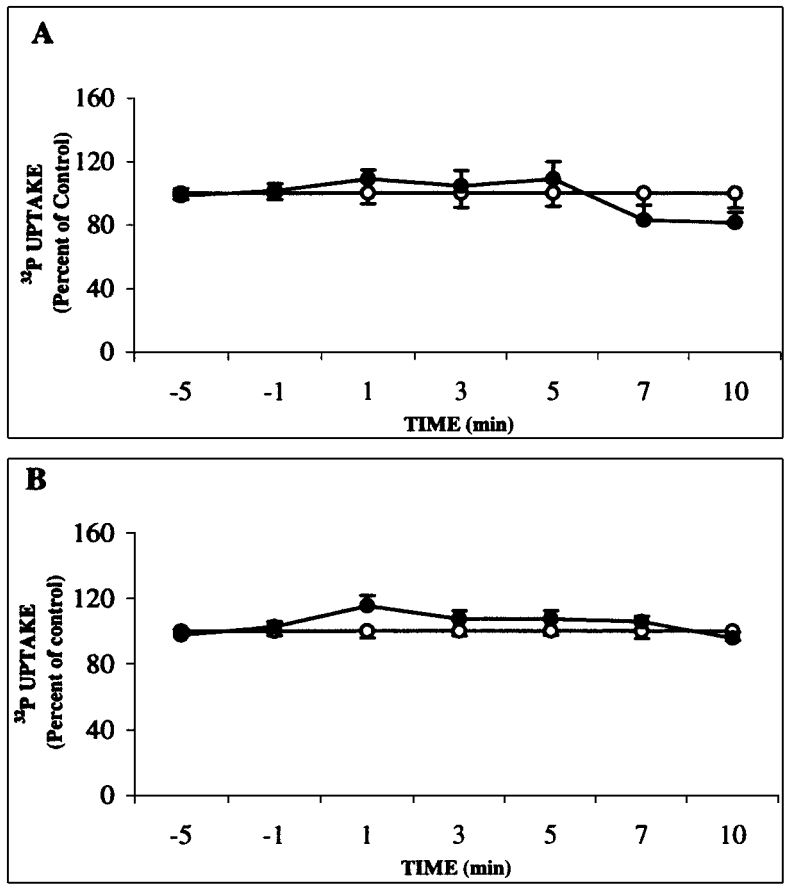

Fig. 8. Effect of 130 pM 1,25(OH)$_2$D$_3$ on phosphate uptake in isolated intestinal cells of (**A**) 14 week birds, n = 5; or (**B**) 28 week birds, n = 4. Procedures were the same as those described in Figure 1. Values represent means ± SEM.

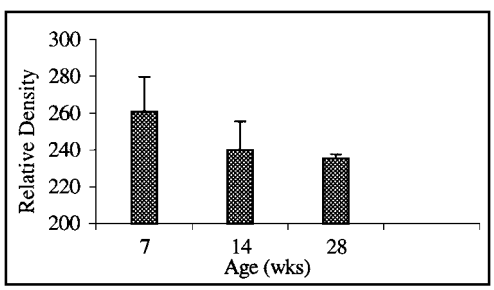

Fig. 9. Densitometric analyses of Westerns of 1,25(OH)$_2$D$_3$-MARRS binding protein in birds of different age groups. BLM were prepared from the indicated age groups and stored at − 20°C until used. Electrophoresis was performed (15 μg of protein per well) on 8% SDS–polyacrylamide gels with colored molecular weight markers as standards. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes, and probed with Ab 099 followed by alkaline-phosphatase conjugated secondary antibody and chromogenic substrate. The bands were quantitated densitometrically. Values represent means ± SEM for three independent experiments.

characteristic common to membrane receptors [Nemere, 1996a, and reference therein].

A further similarity was observed in that the vitamin D$_3$ metabolite 24,25(OH)$_2$D$_3$ inhibited the rapid 1,25(OH)$_2$D$_3$-mediated stimulation of phosphate uptake or transport in both isolated cells or perfused duodenal loops, respectively. The attenuation mediated by 24,25(OH)$_2$D$_3$ is in agreement with the hypothesis that this metabolite is a natural inhibitor of 1,25(OH)$_2$D$_3$ [Nemere, 1999; for review, see Larsson and Nemere, 2001; Farach-Carson and Nemere, 2002; Nemere et al., 2002].

A final point of similarity between the two-model systems is apparent in signal transduction pathways. Forskolin had no effect on phosphate uptake in isolated cells or transport in perfused duodena, suggesting that the PKA pathway is not involved in movement of this ion. In contrast, PKA has been implicated in calcium

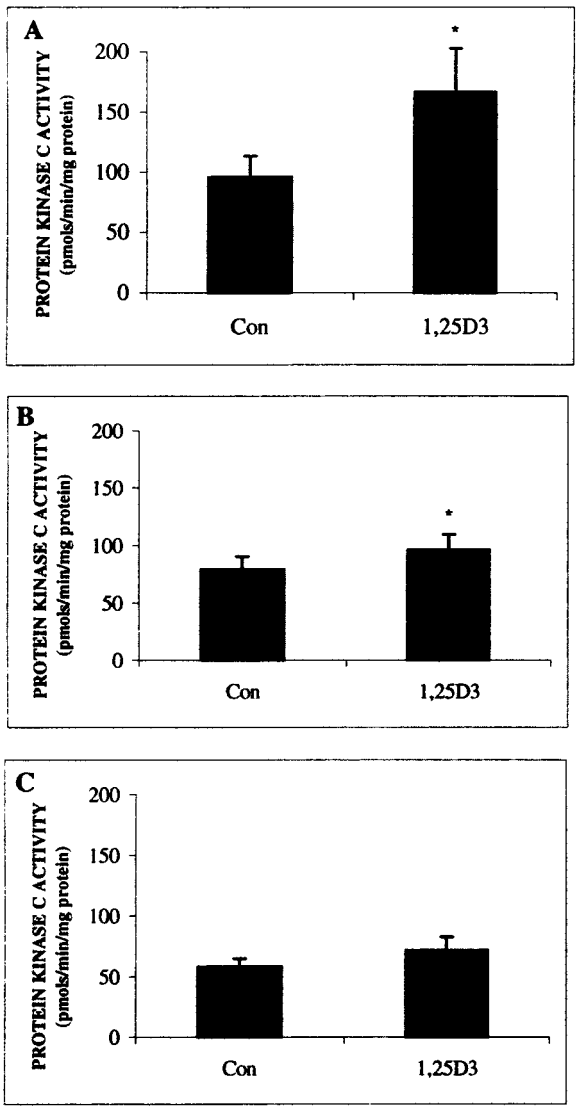

Fig. 10. Effect of 130 pM $1,25(OH)_2D_3$ on PKC activity in intestinal cells isolated from birds of increasing ages. Chick intestinal cells were isolated by chelation from (**A**) 7-, (**B**) 14-, or (**C**) 28-week birds, resuspended in GBSS, and then treated with vehicle or 130 pM $1,25(OH)_2D_3$ for 5 min. Cells were collected by centrifugation and the pellets stored at $-20°C$. Pellets from all age groups were extracted on the same day for determination of PKC activity as described in the text. Values represent means ± SEM for six independent experiments. $*P \leq 0.05$, relative to corresponding controls.

movement in perfused duodena [de Boland and Norman, 1990a], and isolated chick intestinal cells (Nemere, unpublished communications).

PKC is apparently involved in phosphate handling in two tissues, kidney, and intestine. Park et al. [2001] reported that cortisol-mediated inhibition of phosphate uptake is mediated by the PKC signal transduction pathway in primary rabbit kidney proximal tubule cells, whereas the PKA pathway was not involved. In the current report, phorbol ester stimulates phosphate uptake in isolated chick intestinal cells, as well as transport in perfused duodena. In addition, the rapid time course of stimulation by either phorbol ester or $1,25(OH)_2D_3$ are in good agreement: for each agent increased phosphate uptake was evident within 1 min of treatment, and sustained for the entire 10-min time course. It has previously been reported that $1,25(OH)_2D_3$ stimulates PKC activity within the same time frame [Nemere, 1999].

The calcium channel activator, BAY K8644, also stimulates both phosphate uptake in chick enterocytes and transport in perfused duodena when presented to the basal lateral membrane. Work by others [de Boland and Norman, 1990b] has shown that phorbol esters stimulate increased intracellular calcium, as judged by fura-2 fluorescence. In the present work, we found that BAY K8644 did not stimulate PKC activity, suggesting that for signal transduction by $1,25(OH)_2D_3$, localized stimulation of PKC may activate a calcium channel.

The subcellular site(s) of action for the two signal transduction pathways involved in $1,25(OH)_2D_3$-stimulated phosphate handling remain to be determined, although some possibilities are evident. Phosphate uptake in the small intestine occurs through Na/Pi cotransporters [Murer et al., 2000]. Activity might be increased by modification of existing transporters, or vesicular delivery of additional transporters. Once inside the cell, phosphate is taken up into lysosomal vesicles [Wasserman, 1981; Pisoni, 1991; Pisoni and Lindley, 1992; Nemere, 1996b], which is stimulated by $1,25(OH)_2D_3$ in chick intestine [Nemere, 1996b]. Finally, enhanced exocytosis of vesicular contents to complete the transport process may be under hormonal control.

It has been established in rats and humans that intestinal calcium transport declines with age, but the exact mechanisms are still not known. While animals are growing, large amounts of dietary calcium and phosphate are required to build bones, while the adult animal would have less need of rapidly stimulated intestinal uptake. Thus, the rapid effect of $1,25(OH)_2D_3$ on intestinal phosphate uptake, mediated through the $1,25D_3$-MARRS protein, would be expected to decrease if indeed

this pathway is physiologically relevant. The current work indicates a decrease of ^{32}P uptake in the isolated intestinal cells with age, suggesting an involvement of the rapid response pathway. Further, SDS–PAGE followed by Western analysis on isolated BLM showed a decreased expression of the 1,25D$_3$-MARRS protein with increasing age, supporting the results obtained at the cellular level. In addition, as an indicator of receptor-mediated hormone action, PKC activation has been reported to occur in response to 1,25(OH)$_2$D$_3$ in a variety of systems [for review, see Farach-Carson and Nemere, 2002]. In the present work, 130 pM 1,25(OH)$_2$D$_3$ was observed to mediate an approximately twofold increase in PKC activity within 5 min, relative to control incubations in enterocytes from 7-week chicks. However, there was a decrease of PKC activity with increasing ages, which is paralleled by the diminished ^{32}P uptake in isolated intestinal cells and decreasing levels of the expression of the 1,25D$_3$-MARRS binding protein.

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REFERENCES

Armbrecht HJ. 1990. Effect of age on calcium and phosphate absorption. Role of 1,25-dihydroxyvitamin D. Miner Electrolyte Metab 16:159–166.

de Boland AR, Norman A. 1990a. Evidence for involvement of protein kinase C and cyclic adenosine 3′,5′ monophosphate-dependent protein kinase in the 1,25-dihydroxyvitamin D$_3$-mediated rapid stimulation of intestinal calcium transport, (transcaltachia). Endocrinology 127: 39–45.

de Boland AR, Norman AW. 1990b. Influx of extracellular calcium mediates 1,25-dihydroxyvitamin D$_3$-transcaltachia (the rapid stimulation of duodenal Ca^{2+} transport). Endocrinology 127:2475–2480.

Farach-Carson MC, Nemere I. 2002. Membrane receptors for vitamin D steroid hormones: Potential new drug targets. Curr Drug Targets (in press).

Karsenty G, Lacour B, Ulmann A, Pierandrei E, Drueke T. 1985. Early effects of vitamin D metabolites on phosphate fluxes in isolated rat enterocytes. Am J Physiol 248:G40–G45.

Kraft AS, Anderson WB. 1983. Phorbol esters increase the amount of Ca^{2+}, phospholipid-dependent protein kinase associated with plasma membrane. Nature 301:621–631.

Larsson D, Nemere I. 2001. 24,25-Dihydroxyvitamin D$_3$ and intestinal calcium transport: From receptors to physiological response. Rec Res Dev Endocrinol 2(part 1): 183–198.

Murer H, Hernando N, Forster I, Biber J. 2000. Proximal tubular phosphate reabsorption: Molecular mechanisms. Physiol Rev 80:1373–1409.

Nemere I. 1996a. Apparent nonnuclear regulation of intestinal phosphate transport: Effects of 1,25-dihydroxyvitamin D$_3$, 24,25-dihydroxyvitamin D$_3$, and 25-hydroxyvitamin D$_3$. Endocrinology 137:2254–2261.

Nemere I. 1996b. Parathyroid hormone rapidly stimulates phosphate transport in perfused duodenal loops of chicks: Lack of modulation by vitamin D metabolites. Endocrinology 137:3750–3755.

Nemere I. 1999. 24,25-dihydroxyvitamin D$_3$ suppresses the rapid actions of 1,25-dihydroxyvitamin D$_3$ and parathyroid hormone on calcium transport in chick intestine. J Bone Miner Res 14:1543–1549.

Nemere I, Campbell K. 2000. Immunochemical studies on the putative plasmalemmal receptor for 1, 25-dihydroxyvitamin D$_3$. III. Vitamin D status. Steroids 65:451–457.

Nemere I, Farach-Carson MC. 1998. Membrane receptors for steroid hormones: A case for specific cell surface binding sites for vitamin D metabolites and estrogens. Biochem Biophys Res Commun 248:443–449.

Nemere I, Szego CM. 1981a. Early actions of parathyroid hormone and 1,25-dihydroxycholecalciferol on isolated epithelial cells from rat intestine. I. Limited lysosomal enzyme release and calcium uptake. Endocrinology 108: 1450–1462.

Nemere I, Szego CM. 1981b. Early actions of parathyroid hormone and 1,25-dihydroxycholecalciferol on isolated epithelial cells from rat intestine. II. Analyses of additivity, contribution of calcium, and modulatory influence of indomethacin. Endocrinology 109:2180–2187.

Nemere I, Dormanen MC, Hammond MW, Okamura WH, Norman AW. 1994. Identification of a specific binding protein for 1 alpha, 25-dihydroxyvitamin D$_3$ in basal-lateral membranes of chick intestinal epithelium and relationship to transcaltachia. J Biol Chem 269:23750–23756.

Nemere I, Ray R, McManus W. 2000. Immunochemical studies on the putative plasmalemmal receptor for 1,25(OH)$_2$D$_3$. I. Chick intestine. Am J Physiol Endocrinol Metab 278:E1104–E1114.

Nemere I, Safford SE, DeSouza MM, Farach-Carson MC. 2001. Characterization of 1,25D$_3$-membrane-associated rapid response, steroid binding (1,25D$_3$-MARRS) protein from the domestic chicken. Mol Biol Cell 12 (Suppl):331a.

Nemere I, Yazzie-Atkinson D, Johns DO, Larsson D. 2002. Biochemical characterization and purification of a binding protein for 24,25-dihydroxyvitamin D$_3$ from chick intestine. J Endocrinol 172:211–219.

Park S, Taub M, Han H. 2001. Regulation of phosphate uptake in primary cultured rabbit renal proximal tubule cells by glucocorticoids: Evidence for nongenomic as well as genomic mechanisms. Endocrinology 142:710–720.

Pietras RJ, Nemere I, Szego CM. 2001. Cell membrane forms of steroid hormone receptors. Endocrine 14:417–427.

Pisoni RL. 1991. Characterization of a phosphate transport system in human fibroblast lysosomes. J Biol Chem 266:979–885.

Pisoni RL, Lindley ER. 1992. Incorporation of [^{32}P]orthophosphate into long chains of inorganic polyphosphate within lysosomes of human fibroblasts. J Biol Chem 267:3626–3631.

Seamon K, Daly JW. 1981. Activation of adenylate cyclase by the diterpene forskolin does not require the guanine nucleotide regulatory protein. J Biol Chem 256:9799–9801.

Wasserman RH. 1981. Intestinal absorption of calcium and phosphorus. Fed Proc 40:68–72.