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ARTICLES

Effect of Growth and Maturation on Membrane-Initiated Actions of 1,25-Dihydroxyvitamin D_3—II: Calcium Transport, Receptor Kinetics, and Signal Transduction in Intestine of Female Chickens

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Abstract We recently reported (Larsson and Nemere [2003]: Endocrinology 144:1726) the effects of growth and maturation on $1,25(OH)_2D_3$-membrane initiated effects in the intestine of male chickens. Here we extend our observations to studies on females with two stages of high calcium demand: growth (7–14 weeks) and egg laying (28–58 weeks). The rapid stimulatory effect of 130 pM $1,25(OH)_2D_3$ on calcium transport was assessed as a physiological response in perfused duodena of 7-, 14-, 28-, and 58-week-old chickens, and determined to be 308%, 184%, 170%, and 153%, respectively, of corresponding controls after 40 min. Saturation analyses of $[^3H]1,25(OH)_2D_3$ binding to nuclear vitamin D receptor (VDR) indicated an absence of cooperative binding, no changes in dissociation constant (K_d) with age, and an increase in maximum binding capacity (B_{max}) between 7-week birds and older age groups. Analyses of saturable binding of $[^3H]1,25(OH)_2D_3$ to the membrane associated rapid response steroid binding protein ($1,25D_3$-MARRS bp) in basal lateral membranes (BLM), indicated cooperative binding, and an increase in both B_{max} and K_d with age. No changes in the age-related expression of $1,25D_3$-MARRS bp were found, as judged by Western analyses, suggesting that a shift in ligand binding to lower affinity membrane components accounted for the increase in calculated B_{max}. Basal levels of protein kinase C (PKC) activity decreased with age, as did hormone enhancement of activity. Basal levels of protein kinase A (PKA) activity remained constant with age, while the magnitude of hormone stimulation increased. Comparison of dose-response curves for ion transport and kinase activities in 7-week chicks suggested that PKC mediates phosphate transport while PKA mediates calcium transport. Thus, the age-related loss of calcium transport is most likely related to loss of PKC-mediated phosphate transport. J. Cell. Biochem. 90: 901–913, 2003. © 2003 Wiley-Liss, Inc.

Key words: vitamin D; membrane receptors; nuclear receptors; protein kinase C; protein kinase A

The hormonally active steroid 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] is known to elicit effects by two complementary pathways: nuclear-initiated steroid signaling (NISS) and membrane-initiated steroid signaling (MISS). In the intestine, transcriptional effects of $1,25(OH)_2D_3$ induce components of the calcium transport pathway [Nemere, 1996a], which can be rapidly activated by ligand interaction with a putative membrane receptor, first postulated to exist by Nemere and Szego [1981].

Aging is known to impair intestinal calcium absorption [Avioli et al., 1965; Alevizaki et al., 1973; Armbrecht et al., 1980; Armbrecht, 1986; Mooradian and Song, 1989; Wood et al., 1989; Ebeling et al., 1992; Kinyamu et al., 1997], transcriptional effects of $1,25(OH)_2D_3$ [Armbrecht et al., 1980; Liang et al., 1989, 1994; Horst et al., 1990; Takamoto et al., 1990; Cai et al., 1993; Haussler et al., 1998; Strugnell and DeLuca, 1998], and certain MISS pathways [Massheimer et al., 1995; de Boland et al., 1996; Balogh et al., 1997]. In a recent study [Larsson and Nemere, 2003], we found that rapid, $1,25(OH)_2D_3$-stimulated calcium transport in isolated, perfused duodena decreased with

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age in male chickens. This altered physiological response was accompanied by changes in binding parameters of the $1,25(OH)_2D_3$ membrane associated, rapid response steroid ($1,25\text{-}D_3$-MARRS) binding protein (bp) and signal transduction. We concluded that the $1,25(OH)_2D_3$-regulated MISS pathways are important in supporting growth.

In the current report, we assess whether similar growth-related phenomena occur in female chickens, and further, whether the calcium demands of egg-laying involve the participation of $1,25(OH)_2D_3$ MISS.

MATERIALS AND METHODS

Materials

Female white Leghorn chicks were from Merrill Poultry, ID, chloropent from Fort Dodge Laboratories (Fort Dodge, IA), $^{45}CaCl_2$ and [^{3}H]$1,25(OH)_2D_3$ were from NEN Life Science Products, Inc. (Boston, MA). Immobilon-P polyvinylidene difluoride (PVDF) membranes were from Millipore (Bradford, MA), kits for protein kinase C (PKC) and protein kinase A (PKA) determination were from Life Technologies-GIBCO (Waverly, MA), Bradford dye was from Bio-Rad (Hercules, CA), and [γ-^{32}P]ATP was purchased from NEN (Boston, MA). All other chemicals were of highest grade available and obtained from Sigma Chemicals Co. (St. Louis, MO).

Animals

White Leghorn pullets were obtained on the day of hatch and raised on a vitamin D-supplemented diet prior to experimentation. Animals of ages 7 and 14 weeks were raised on a standard growth diet containing 1% calcium and 1% phosphorous. Hens 28–58 weeks of age were raised on an isocaloric diet containing 5% calcium and 5% phosphorous to meet the demands of egg-laying, and prevent hypocalcemia with the attendant shifts in circulating calcitropic hormone levels. On the day of the experiments, chickens were anesthetized with 0.3 ml/100g B.W. chloropent. All protocols were approved by the Utah State University Institutional Animal Care and Use Committee.

Perfusion Studies

Perfusion studies were performed as described earlier [Nemere, 1996b, 1999; Larsson and Nemere, 2003]. In brief, the vascular perfusion medium was Gey's balanced salt solution (GBSS; 119 mM NaCl, 4.96 mM KCl, 0.22 mM KH_2PO_4, 0.84 mM Na_2HPO_4, 1.03 mM $MgCl_2$, 0.28 mM $MgSO_4$, 0.9 mM $CaCl_2$, 5 mM glucose, 27 mM bicarbonate and aerated with 95% O_2/5% CO_2). A side pump delivered bovine serum albumin (BSA; 0.125% w/v final concentration) and either metabolite or the vehicle ethanol (0.005%, final concentration). The luminal perfusion medium lacked bicarbonate and glucose, and contained 1 μCi/ml $^{45}CaCl_2$. Each perfusion experiment was divided into three periods. During the first period (10 min), vascular perfusion with control medium (containing 0.005% ethanol, final concentration) proceeded to allow the system to reach a steady state. During the second period (10 min), fractions were collected for assessment of basal ^{45}Ca transport. Throughout the third period, the preparation was exposed to either GBSS-control medium or 130 pM $1,25(OH)_2D_3$ for 40 min and radioactivity determined in the collected fractions. The transport during the third period was normalized to the corresponding average basal transport rate.

Preparation of Crude Nuclei and Basal Lateral Membranes (BLM)

Subcellular fractions were prepared as reported earlier [Nemere et al., 1986, 1994; Larsson and Nemere, 2003]. Intestinal epithelium was disrupted in 40 ml homogenization medium (250 mM sucrose, 5 mM histidine-imidazole, 2 mM EGTA, pH 7.0) with a Dounce homogenizer and a Teflon pestle. The epithelium was fractioned by differential centrifugation, resulting in a crude nuclear fraction, and a fraction containing intracellular organelles plus BLMs. Further separation of the organelles and membranes was achieved by centrifugation in Percoll. Eighteen fractions were collected from the gradient (52 drops each). Fractions 16–18 containing BLM [Nemere et al., 1986, 1994; Larsson and Nemere, 2003] were pooled and the Percoll removed by ultracentrifugation [Nemere et al., 1994; Larsson and Nemere, 2003]. Three independent preparations of crude nuclei and BLM from each age group were stored at −20°C until used.

Saturation Analysis of [^{3}H]$1,25(OH)_2D_3$ Binding

BLM fractions were adjusted to 50 μg of protein per tube and incubated in TED buffer

(10 mM Tris, 2 mM EDTA, 1 mM dithiothreitol, pH 7.4) overnight (0°C) with 0.5, 1.0, 2.0, or 4.0 nM [^{3}H]1,25(OH)$_2$D$_3$ in the absence (total binding) or in the presence of a 200-fold molar excess of unlabeled 1,25(OH)$_2$D$_3$ (nonspecific binding). Each sample was assayed in triplicate for both total and nonspecific binding. Nuclear fractions from each group were incubated in the same way as BLMs, but with [^{3}H]1,25(OH)$_2$D$_3$ concentrations of 0.05, 0.1, 0.25, 0.5, 1.0, or 2.0 nM.

After incubation overnight, bound and free ligands were separated by either perchloric acid precipitation, for binding to BLM [Larsson and Nemere, 2003] or by a hydroxylapatite (HAP) assay for binding to nuclear fractions [Wecksler and Norman, 1979; Larsson and Nemere, 2003].

For BLM fractions, perchloric acid and carrier protein (γ-globulin) were added to each tube, the mixture was incubated on ice for 20 min, and the precipitated protein pelleted by centrifugation. The pellets containing the seco-steroid binding moiety were solubilized in guanidinium/HCl, decanted into vials, liquid scintillation cocktail added, and radioactivity determined.

While the perchloric acid method is suitable for hydrophobic membrane proteins (1,25D$_3$-MARRS bp), it fails to detect the soluble classical vitamin D receptor (nVDR) [Nemere et al., 1994]. Binding of [^{3}H]1,25(OH)$_2$D$_3$ was therefore assessed in crude preparations of nuclei by the use of a HAP assay as reported elsewhere [Wecksler and Norman, 1979]. After incubation with different concentrations of [^{3}H]1,25(OH)$_2$D$_3$ overnight (see above), 200 μl HAP and 800 μl TED containing 0.5% Triton X-100 were added to each tube, and mixed. The HAP-bound receptor was pelleted by centrifugation, washed twice with 800 μl TED containing 0.5% Triton X-100, treated with 1 ml ethanol to extract ligand, and centrifuged. The resulting supernatant fraction was transferred to a glass scintillation vial, the ethanol evaporated and the amount [^{3}H]1,25(OH)$_2$D$_3$ measured after addition of fluor.

SDS–PAGE and Western Analysis

SDS–PAGE and Western blot analysis were used to determine immunoreactive levels of 1,25D$_3$-MARRS bp as previously described [Nemere et al., 2000; Larsson and Nemere, 2003].

PKC and PKA Activity and Protein Determination

Intestinal epithelial cells from two duodena per experiment were isolated by citrate chelation as reported elsewhere [Nemere and Campbell, 2000]. After removal of the duodenal loop and chilling for 15 min in saline, the pancreas was excised, the duodenum was everted, rinsed in saline, and the intestinal epithelial cells isolated by stirring the segments in chelation media for three, 15-min periods. The isolated cells were combined, centrifuged at 500*g*, 4°C for 5 min and the resulting pellet resuspended in 20 ml GBSS-BSA (0.125% BSA, w/v). Cells (100 μl aliquots) were treated at room temperature with either ethanol (0.005%, final concentration) or the indicated concentrations of 1,25(OH)$_2$D$_3$ at time zero. For PKC, cells were incubated for 5 min, and for PKA the incubation lasted for 7 min [Nemere, 1999; Larsson and Nemere, 2003]. After incubation, the cells were centrifuged at 1,000*g*, 4°C for 10 min, the supernatant decanted, and the pellet stored at −20°C until the day of use.

PKC and PKA activity were analyzed with commercially available assay systems. The analyses and activity calculations were performed according to instructions packaged with the kits [Larsson and Nemere, 2003]. Briefly, enzyme activity was extracted by homogenizing treated cells in the appropriate extraction buffer followed by incubation on ice for 30 min and centrifugation. Supernatants were analyzed for PKA or PKC activity. For PKC activity 10 μg of protein were incubated at room temperature for 20 min in the presence of PKC activator or inhibitor. [γ-^{32}P]ATP (20-25 μCi/ml) was added to each tube, incubated for 5 min at 30°C, and 25 μl of the mixture spotted onto a phosphocellulose disc. The discs were washed, transferred to scintillation vials, incorporated ^{32}P assessed, and specific PKC activity calculated. For PKA activity, proteins (5 μg) were incubated at room temperature for 20 min in the presence of activator or inhibitor only, in the presence of both activator and inhibitor and in the absence of both. [γ-^{32}P]ATP (20–25 μCi/ml) was added to each tube, incubated for 5 min at 30°C, and 20 μl of the mixture spotted onto phosphocellulose disc. The discs were washed, transferred into scintillation vials, ^{32}P assessed, and PKA activity calculated.

Proteins were determined using the Bradford dye (BioRad), according to manufacturer's instructions.

Statistics and Data Analysis

The specific binding of [^{3}H]1,25(OH)$_2$D$_3$ to receptor was calculated and plotted against the corresponding concentration of [^{3}H]1,25-(OH)$_2$D$_3$ The data were analyzed by nonlinear regression analysis by fitting either to a three parameter sigmoid equation or a hyperbolic function.

The sigmoid equation was as follows:

$$Y = \frac{a}{1 + e^{-\left(\frac{x - x_0}{b}\right)}} \quad (1)$$

where Y is specifically bound [^{3}H]1,25(OH)$_2$D$_3$ (fmol/mg protein), x is the concentration of 1,25(OH)$_2$D$_3$ (nM) in the incubation mixture, a is the maximum specifically bound [^{3}H]1,25-(OH)$_2$D$_3$ (B_{max}), b is the minimum specifically bound [^{3}H]1,25(OH)$_2$D$_3$, and x_0 is the concentration of 1,25(OH)$_2$D$_3$ in the incubation mixture at half maximum bound [^{3}H]1,25(OH)$_2$D$_3$ (K_d).

For the hyperbolic function, the equation was as follows:

$$Y = \frac{a * x}{b + x} \quad (2)$$

where Y is specifically bound [^{3}H]1,25(OH)$_2$D$_3$ (fmol/mg protein), x is the concentration of 1,25(OH)$_2$D$_3$ (nM) in the incubation mixture, a is the maximum specifically bound [^{3}H]1,25-(OH)$_2$D$_3$ (B_{max}), and b is the concentration of 1,25(OH)$_2$D$_3$ in the incubation mixture at half maximal bound [^{3}H]1,25(OH)$_2$D$_3$ (K_d).

Data from Hill analysis, perfusion studies, and PKA and PKC activities were analyzed by linear regression. The coefficient of variation (R^2) and the adjusted variation (adjR^2) were used as a measure of how well the regression model describes the data. A one-way analysis of variance (ANOVA) with F-statistics was used to gauge the contribution of the independent variable to predict the dependent variable. The significance level was set at $P < 0.05$, and data are presented as mean ± SEM.

When comparing B_{max}, K_d, Hill coefficients, and changes in calcium transport over time within and between different age groups, an unpaired Student's t-test with adjustment for mass significance was used. In the PKC, PKA, and Western blot analysis, statistical comparisons were performed using one-way ANOVA followed by Student's Newman–Keuls posthoc test when comparing a factor with more than two levels. The tests used were two-tailed, and the significance level was set at $P < 0.05$. Data are presented as mean ± SEM.

RESULTS

Effects of Age on Intestinal Calcium Transport

As a test of physiological responsiveness to hormone, calcium transport was studied in isolated, perfused duodena. Female chickens of ages 7, 14, 28, and 58 weeks having average weights (mean kg ± SEM) of 0.33 ± 0.06, 0.91 ± 0.02, 1.47 ± 0.06, and 1.71 ± 0.04, respectively, were used. For stimulation of calcium transport, 130 pM 1,25(OH)$_2$D$_3$ was used in the vascular perfusate, and compared to vehicle controls. As shown in Figure 1A–D, treatment with 130 pM 1,25(OH)$_2$D$_3$ resulted in a linear increase in intestinal calcium transport which was significantly ($P < 0.0125$) higher than that observed in vehicle-treated preparations for all ages tested. However, a significant ($P < 0.0125$) age dependent decrease in stimulated calcium transport was observed between 7- and 14-week-old birds when comparing slope coefficients (Fig. 1A). Steroid-stimulated transport at 40 min went from 308% of controls for 7-week birds to 184% for 14-week birds. In addition, 28-week-old birds showed a significantly higher calcium transport after 40 min of hormone (170% of controls; Fig. 1C) compared to 58-week-old birds (153% of controls; Fig. 1D). Basal transport values (cpm/0.1 ml) for 7-, 14-, 28-, and 58-week birds were, respectively, 198 ± 25, 152 ± 24, 308 ± 32, and 149 ± 25. Values for preparations from egg-laying 28-week-old birds were significantly greater than basal transport rates in duodena of other age groups.

In other perfusion studies with year-old hens, neither 65 pM bPTH(1-34) nor 1 nM estradiol 17β were effective in stimulating calcium transport to more than 135% of controls at T = 40 min (data not shown).

Saturation Analysis of 1,25(OH)$_2$D$_3$ Binding to nVDR

Figures 2A–D illustrate the results in which the specific binding activity was determined as a function of increasing concentrations of ligand in nuclear fractions prepared from 7-, 14-, 28-, and 58-week-old female birds (n = 3 for each age

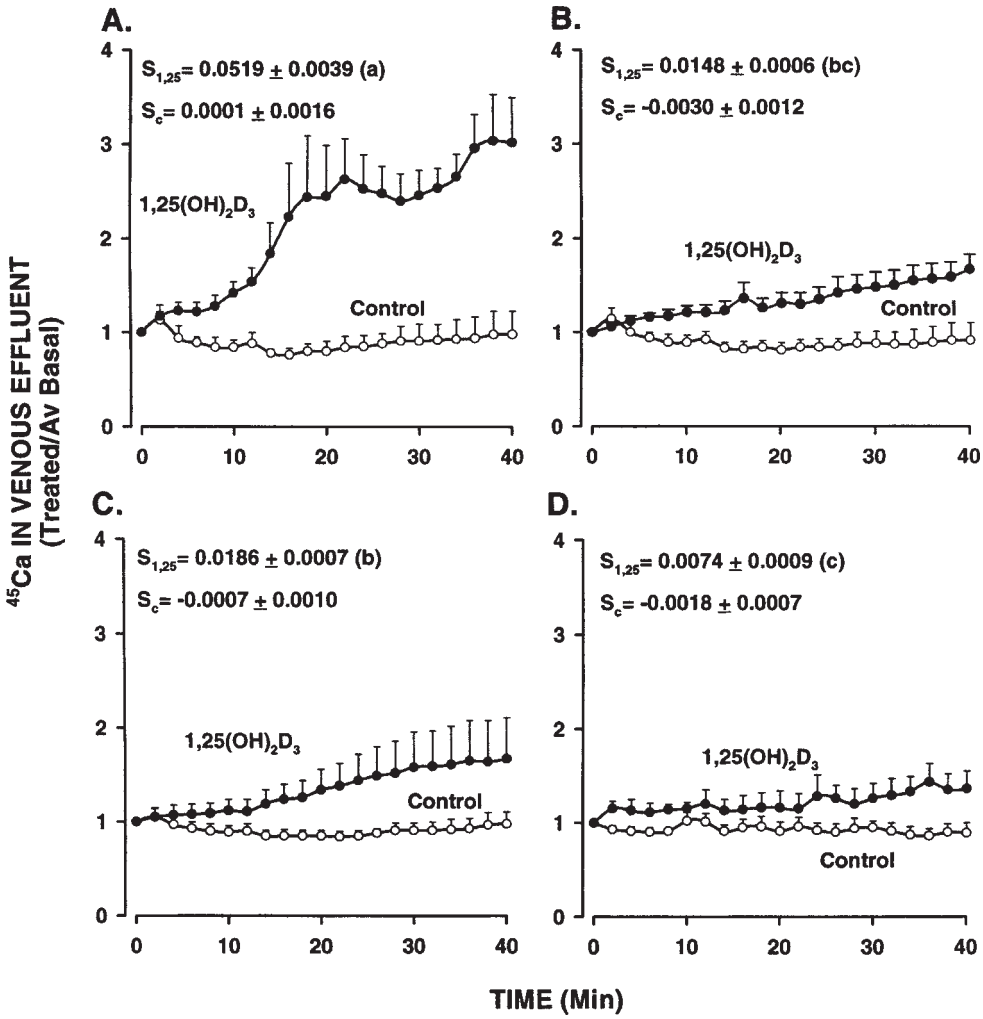

Fig. 1. Effects of age on the rapid stimulation of intestinal calcium transport by 1,25(OH)$_2$D$_3$. Isolated duodena of female chickens of ages (**A**) 7, (**B**) 14, (**C**) 28, and (**D**) 58 weeks were vascularly perfused with vehicle (0.005% ethanol, final concentration) during the basal phase, and again in the treated phase for controls (open symbols), or with 130 pM 1,25(OH)$_2$D$_3$ during the treated phase (closed symbols). Values represent ^{45}Ca in the venous effluent at T min divided by average basal cpm. Data were analyzed by linear regression and the slopes for 1,25(OH)$_2$D$_3$ ($S_{1,25}$) and vehicle treated (S_c) preparations were calculated. Significant differences in the slope coefficients between $S_{1,25}$ and S_c within each age group and between $S_{1,25}$ in different age groups were determined by a two-way ANOVA followed by Student's *t*-test ($P < 0.0125$). All age groups showed a significant ($P < 0.0125$) increase in calcium after treatment with 1,25(OH)$_2$D$_3$ compared to control treatment. Small letters indicate significant differences ($P < 0.0125$) when comparing $S_{1,25}$ between age groups. Data are expressed as mean ± SEM ($n = 4–7$).

group). Data obtained in the analyses were fitted to a hyperbolic function ($P < 0.05$ for all ages, 7 weeks: $F = 150.6$, $DF = 12$, $R^2 = 0.93$, and $adjR^2 = 0.92$; 14 weeks: $F = 115.9$, $DF = 13$, $R^2 = 0.91$, and $adjR^2 = 0.90$; 28 weeks: $F = 30.97$, $DF = 13$, $R^2 = 0.72$, and $adjR^2 = 0.70$; and 58 weeks: $F = 77.3$, $DF = 13$, $R^2 = 0.87$, and $adjR^2 = 0.85$). Maximal binding capacities (B_{max}) for 7-, 14-, 28-, and 58-week-old birds were calculated to be 18.9 ± 1.0, 29.9 ± 2.6, 25.7 ± 3.0, and 28.7 ± 2.5 fmol/mg protein, respectively. The increase in B_{max} between 7-week birds and 14- and 58-week birds was significant ($P < 0.016$). In the same age groups, specific binding became half-saturable at a K_d of 0.08 ± 0.02, 0.20 ± 0.06, 0.06 ± 0.04, and 0.11 ± 0.04 nM, respectively. No significant differences in K_d with increasing age were observed.

Data presented in Figure 2A were recalculated for Hill analyses, which yielded curves that could be described by a linear regression ($P < 0.05$ for all ages, 7 weeks: $F = 33.0$, $DF = 10$, $R^2 = 0.79$, and $adjR^2 = 0.76$; 14 weeks: $F = 42.5$,

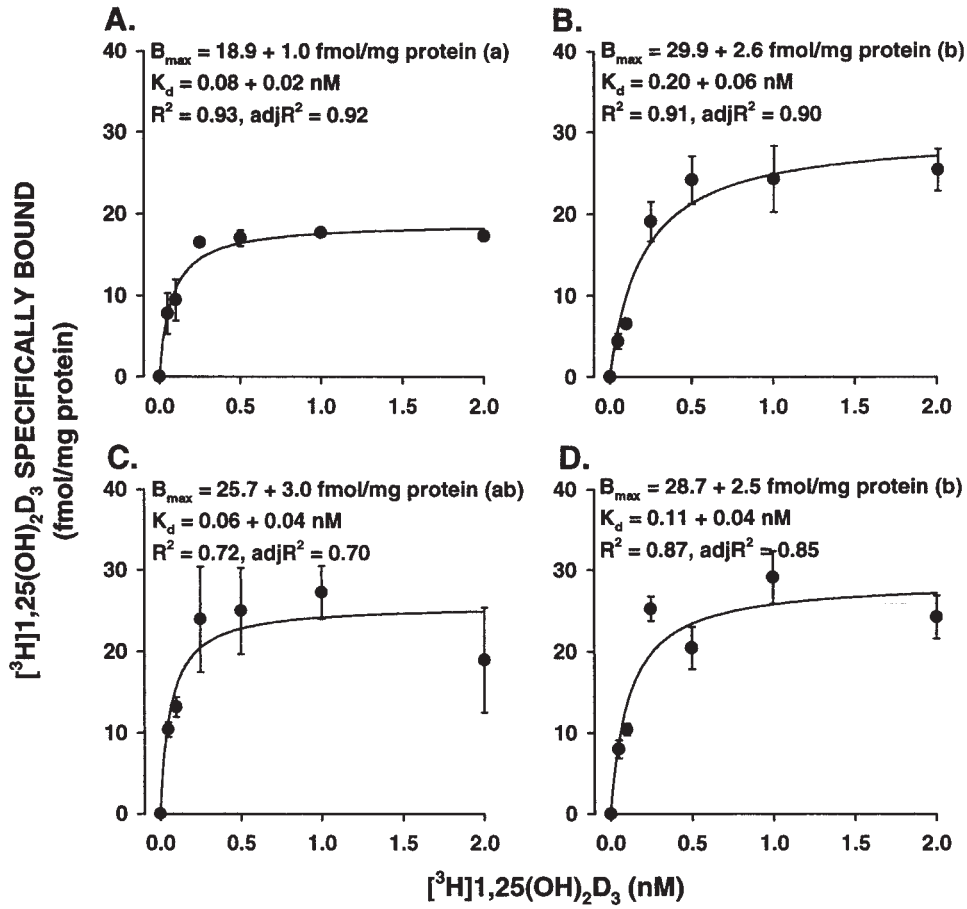

Fig. 2. Saturation analysis of 1,25(OH)$_2$D$_3$ binding to nVDR. Crude nuclear fractions enriched in nVDR isolated from female chickens of ages (**A**) 7, (**B**) 14, (**C**) 28, and (**D**) 58 weeks were incubated with 0.05, 0.1, 0.25, 0.5, 1.0, or 2.0 nM [^{3}H]1,25(OH)$_2$D$_3$ in the absence or in presence of 200-fold molar excess of unlabeled steroid. Bound radioactivity was assessed with the HAP assay. Data were fitted to a three-parameter hyperbolic function and tested by nonlinear regression. R^2, coefficient of variance and adjR^2, the adjusted coefficient of variance. Small letters indicate significant differences determined by a one-way ANOVA followed by Student's *t*-test. $P < 0.016$ for significance and data are presented as mean ± SEM (n = 3 independent preparations). In this and subsequent figures, symbols without error bars indicate SEMs smaller than the closed symbol.

DF = 11, $R^2 = 0.81$, and adj$R^2 = 0.79$; 28 weeks: F = 6.37, DF = 8, $R^2 = 0.88$, and adj$R^2 = 0.86$; and 58 weeks: F = 60.2, DF = 7, $R^2 = 0.91$, and adj$R^2 = 0.89$). The resultant apparent Hill coefficients (n_{app}) were 0.88 ± 0.15, 1.10 ± 0.17, 0.74 ± 0.11, and 0.99 ± 0.13 for 7-, 14-, 28-, and 58-week-old birds, respectively (Fig. 3A). No significant differences in n_{app} were observed for the different ages. Taken together, both the hyperbolic distribution and the slope in the Hill analyses indicate absence of cooperative binding.

Saturation Analysis of 1,25(OH)$_2$D$_3$ Binding to 1,25D$_3$-MARRS bp and Western Analysis

Figures 4A–D illustrate the specific binding activity as a function of increasing concentrations of ligand in BLM fractions prepared from intestinal mucosa of 7-, 14-, 28-, and 58-week-old female birds (n = 3 for each age group). Data obtained in these analyses were fitted to a sigmoid function ($P < 0.05$ and DF = 14 for all ages, 7 weeks: F = 22.1, $R^2 = 0.79$, and adj$R^2 = 0.75$; 14 weeks: F = 41.69, $R^2 = 0.87$, and adj$R^2 = 0.85$; 28 weeks: F = 65.3, $R^2 = 0.92$, and adj$R^2 = 0.90$; and 58 weeks: F = 43.5, $R^2 = 0.88$, and adj$R^2 = 0.85$). B_{max} values were calculated to be 209.4 ± 21.0, 331.5 ± 30.4, 292.3 ± 24.0, and 456.0 ± 43.9 fmol/mg protein, and values of K_d were calculated to be 0.83 ± 0.16, 1.31 ± 0.19, 1.56 ± 0.21, and 1.53 ± 0.22 nM for 7-, 14-, 28-, and 58-week-old birds, respectively. Significant ($P < 0.016$) age-related increases in both K_d and B_{max} were observed, indicating a

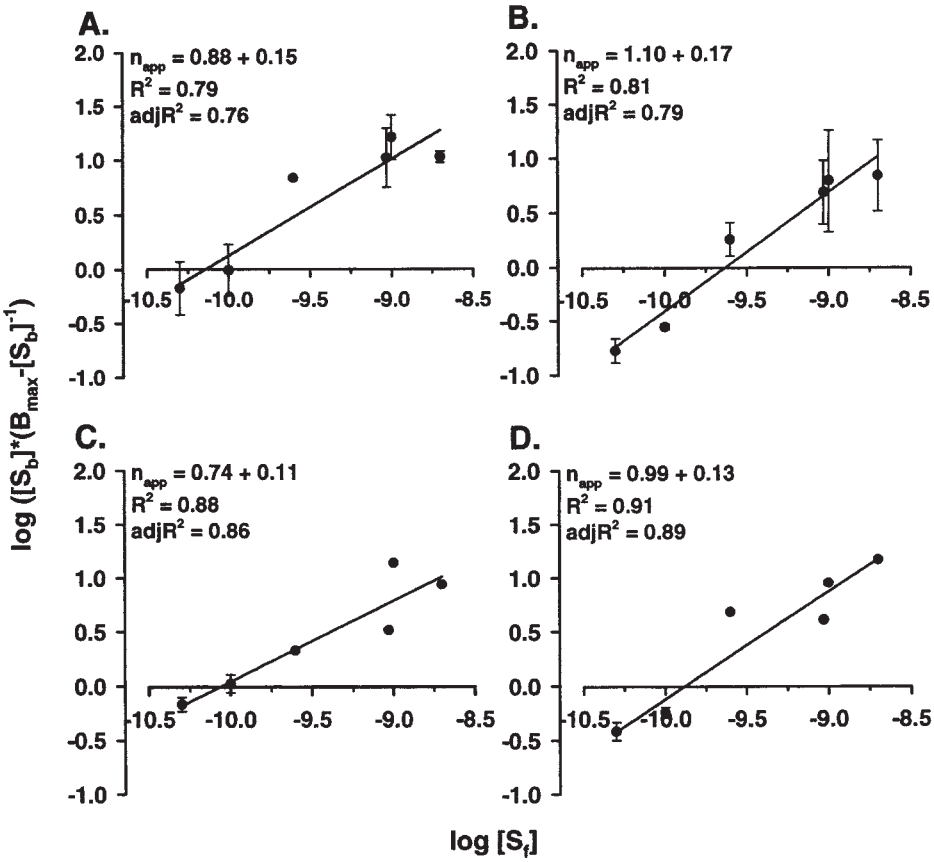

Fig. 3. Hill analysis of 1,25(OH)$_2$D$_3$ binding to nVDR. Data presented in Figure 2A were recalculated for Hill analysis. **A**: 7, (**B**) 14, (**C**) 28, and (**D**) 58-week-old birds. [S$_b$], specifically bound fraction of the administered total free ([S$_f$]) [^{3}H]1,25(OH)$_2$D$_3$; R^2, coefficient of variance; and adjR^2, the adjusted coefficient of variance. No significant differences in apparent Hill coefficient (n_{app}) were observed with increasing age when compared by a one-way ANOVA followed by Student's *t*-test. The n_{app} close to 1 show presence of noncooperative binding for 1,25(OH)$_2$D$_3$ to nVDR. Data are presented as mean ± SEM (n = 3 per tested [^{3}H]1,25(OH)$_2$D$_3$ concentration) and $P < 0.016$ for significance.

reduced affinity and increased number of binding sites in membranes with age.

A recalculation of the data in Figure 4A for Hill analyses resulted in curves that were described by linear regression ($P < 0.05$ for all ages, 7 weeks: F = 33.4, DF = 7, $R^2 = 0.70$, and adj$R^2 = 0.65$; 14 weeks: F = 11.6, DF = 8, $R^2 = 0.62$, and adj$R^2 = 0.57$; 28 weeks: F = 35.5 DF = 9, $R^2 = 0.82$, and adj$R^2 = 0.79$; and 58 weeks: F = 38.3, DF = 9, $R^2 = 0.83$, and adj$R^2 = 0.81$). The Hill analyses yielded Hill coefficients greater than 1 for all ages indicating a presence of positive cooperativity in the binding of 1,25(OH)$_2$D$_3$ to 1,25D$_3$-MARRS bp. The n_{app} were calculated to be 2.64 ± 0.45, 1.24 ± 0.36, 1.67 ± 0.39, and 1.57 ± 0.25 for 7-, 14-, 28-, and 58-week-old birds, respectively (Fig. 5A). Statistical comparison of n_{app} between the ages showed no significant difference.

For each age group, three independent BLM preparations were subjected to SDS–PAGE followed by Western blot analyses of 1,25D$_3$-MARRS bp expression. Densitometric values for BLM prepared from 7-week chicks were averaged and used to normalize values. Relative values for 7-, 14-, 28-, and 58-week birds were, respectively 1.00 ± 0.07, 1.05 ± 0.07, 0.97 ± 0.02, and 0.91 ± 0.06. While the average levels of immunoreactivity tended to decrease with age, the changes were not statistically significant. The age-associated decline in affinity and increase in total binding sites may be due to increased levels of lower affinity components and/or a decrease in heterodimeric binding partners, in analogy to findings in male birds [Larsson and Nemere, 2003].

Effects of Age on 1,25(OH)$_2$D$_3$ Induced PKC and PKA Activity

Both PKC and PKA have been implicated in mediating rapid, steroid-hormone-induced calcium transport [de Boland and Norman,

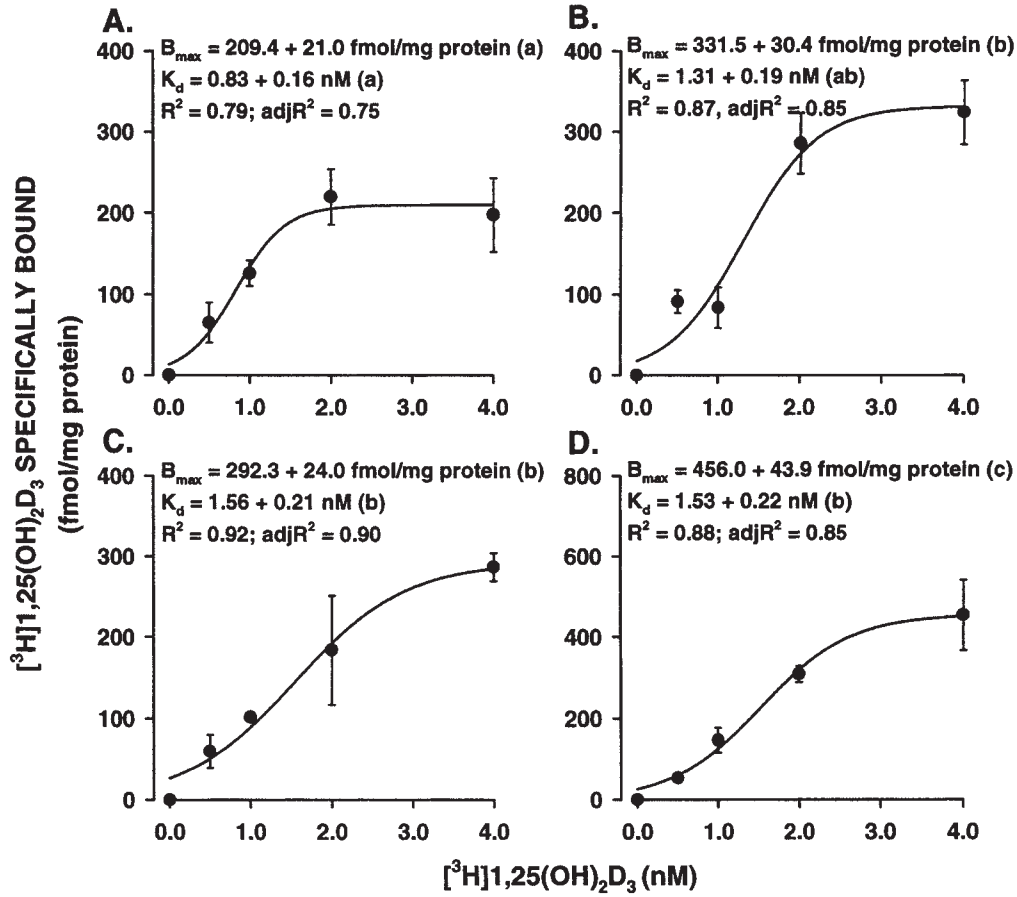

Fig. 4. Saturation analysis of 1,25(OH)$_2$D$_3$ binding to 1,25D$_3$-MARRS bp. Basal lateral membranes (BLM) isolated from female chickens of ages (**A**) 7, (**B**) 14, (**C**) 28, and (**D**) 58 weeks were incubated with 0.5, 1.0, 2.0, or 4.0 nM [^{3}H]1,25(OH)$_2$D$_3$ in the absence or in presence of 200-fold molar excess of unlabeled steroid. Bound radioactivity was assessed by precipitation with perchloric acid in the presence of carrier bovine γ-globulin. Data were fitted to a three-parameter sigmoid function and tested by nonlinear regression. R^2, coefficient of variance and adjR^2, the adjusted coefficient of variance. Significant differences in maximum binding capacity, B_{max}, and dissociation constant, K_d, with increasing age were determined by a one-way ANOVA followed by Student's *t*-test. Small letters indicate significant differences ($P < 0.016$) between ages. Data are presented as mean ± SEM (n = 3 per tested [^{3}H]1,25(OH)$_2$D$_3$ concentration).

1990a]. Since it is plausible that differences in intestinal responsiveness to steroid hormones may be due to pleiotropic changes at the cellular level between growing and mature animals, the effects of 130 pM 1,25(OH)$_2$D$_3$ on PKC and PKA activity in isolated intestinal cells from female chickens of different ages were tested. Table I shows the PKC and PKA activity in both untreated and hormone-treated cells from 7-, 14-, 28-, and 58-week-old birds. Basal PKA activity was unaffected by age as demonstrated by vehicle controls (Table I), while PKC activity showed an apparent increase in basal activity at 14 weeks, which became significant ($P < 0.05$) for 28-week-old birds. Basal PKC activity declined again in 58-week-old birds (Table I). Upon comparison of 1,25(OH)$_2$D$_3$ treated cells with corresponding controls within each age group, a significant ($P < 0.05$) increase in PKC activity was observed in preparations from 7-week-old animals (Table I). Steroid-mediated stimulation of PKC activity was essentially absent in cells prepared from older groups of birds. By comparison, the magnitude of 1,25(OH)$_2$D$_3$-stimulated PKA activity exhibited an age-dependent increase, with significant differences observed in cell preparations from 28- and 58-week birds (Table I). The differences in age-related changes in hormone-stimulated PKC and PKA are also presented in Table I. When expressed as percent of control, a significant ($P < 0.05$) linear decrease in steroid-

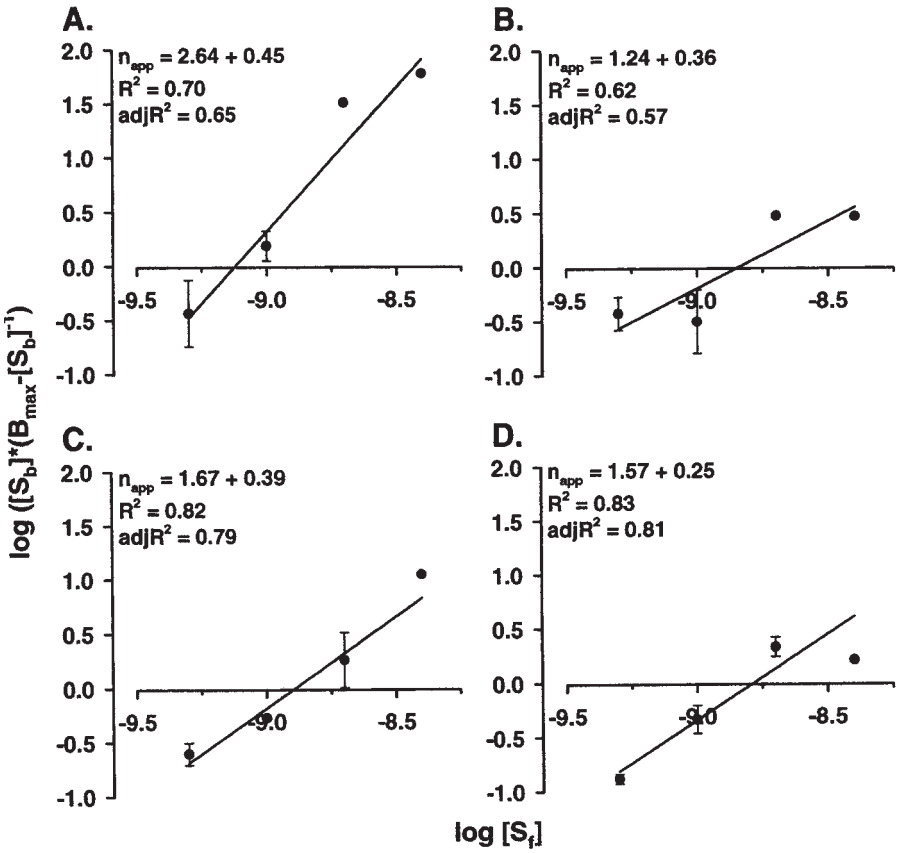

Fig. 5. Hill analysis of 1,25(OH)$_2$D$_3$ binding to 1,25D$_3$-MARRS bp. Data presented in Figure 4A were recalculated for Hill analysis. **A**: 7, (**B**) 14, (**C**) 28, and (**D**) 58-week-old birds. [S_b], specifically bound fraction of the administered total free ([S_f]) [^{3}H]1,25(OH)$_2$D$_3$; R^2, coefficient of variance; and adjR^2, the adjusted coefficient of variance. The apparent Hill coefficients (n_{app}) were larger than 1 in all age groups, indicating positive cooperative binding for 1,25(OH)$_2$D$_3$ to 1,25D$_3$-MARRS bp. No significant differences in apparent Hill coefficient (n_{app}) were observed with increasing age, when compared by a one-way ANOVA followed by Student's *t*-test. Data are presented as mean ± SEM.

enhanced PKC activity was observed with age (Table I), while a significant ($P < 0.05$) linear increase in steroid-enhanced PKA activity was observed as a function of aging (Table I).

To help evaluate these disparate age-related findings, dose-response analyses of PKC and PKA stimulation were undertaken. Figure 6 depicts the results of dose-response analyses for 1,25(OH)$_2$D$_3$-stimulated PKC activity in isolated intestinal epithelial cells of 7-week-old chicks. Near optimal stimulation was observed at 26- and 130-pM seco-steroid (Fig. 6).

TABLE I. Basal and Activated Levels of PKC and PKA Activities as a Function of Age

	PKC activity			PKA activity		
Age (weeks)	Con	1,25D$_3$	% Control	Con	1,25D$_3$	% Control
7	57 ± 6[a]	84 ± 8*	148 ± 8	29 ± 2	47 ± 9	162 ± 13
14	84 ± 13[a]	94 ± 9	120 ± 15	29 ± 2	52 ± 12	179 ± 16
28	132 ± 11[b]	136 ± 12	105 ± 3	21 ± 2	54 ± 8*	257 ± 17
58	70 ± 11[a]	59 ± 3	84 ± 5**	32 ± 12	102 ± 15*	319 ± 30**

Isolated intestinal epithelial cells from female chickens were analyzed for specific activities of PKC (pmol/min/mg protein) or PKA (percent activated PKA/mg protein), after treatment with vehicle (Con) or 130 pM 1,25(OH)$_2$D$_3$ (1,25D$_3$).
[a,b]$P < 0.05$, between age groups.
*$P < 0.05$, relative to corresponding control.
**$P < 0.05$, linear regression analysis between 7-, 14-, 28-, and 58-week age groups.

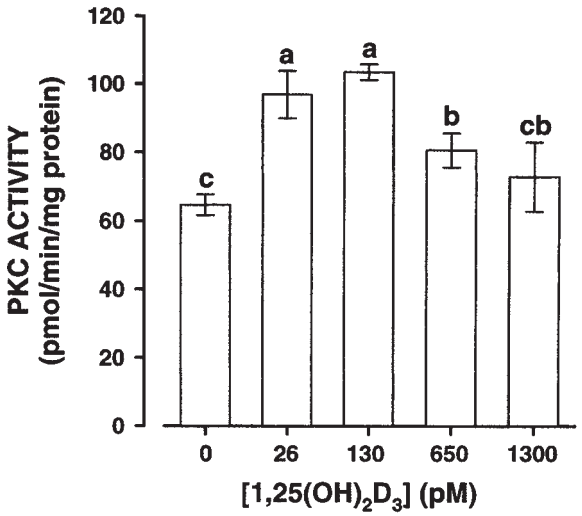

Fig. 6. Dose-response analysis of $1,25(OH)_2D_3$-stimulated PKC activity. Intestinal epithelial cells were isolated from 7-week-old chicks by citrate chelation, collected by centrifugation, and resuspended in GBSS-0.1% BSA. At zero time, cells were treated either with vehicle or the indicated concentrations of $1,25(OH)_2D_3$ for 5 min. Cells were then harvested by centrifugation (4°C), extracted and analyzed for PKC activity and protein. Values represent mean ± SEM for 0, 130, and 650 pM $1,25(OH)_2D_3$ (n = 3), and mean ± range for 26 and 1,300 pM steroid (n = 2). Lower case letters (a, b) indicate significant differences ($P < 0.05$) between specific activities obtained for different hormone concentrations.

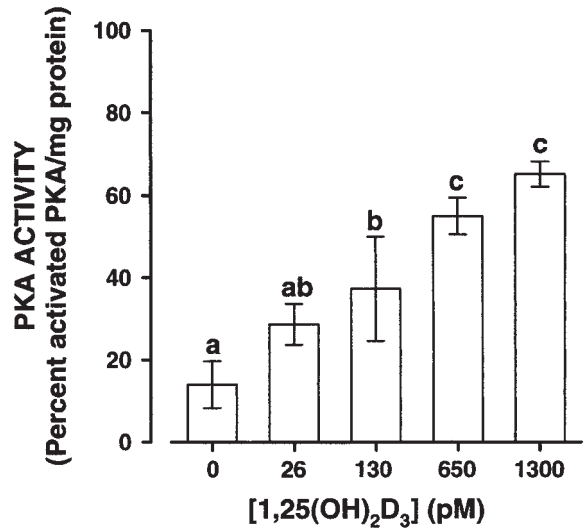

Fig. 7. Dose-response analysis of $1,25(OH)_2D_3$-stimulated PKA activity. Intestinal epithelial cells were isolated from 7-week-old chicks by citrate chelation, collected by centrifugation, and resuspended in GBSS-0.1% BSA. At zero time, cells were treated either with vehicle or the indicated concentrations of $1,25(OH)_2D_3$ for 7 min. Cells were then harvested by centrifugation (4°C), extracted and analyzed for PKA activity and protein. Values represent mean ± SEM for 0, 130, and 650 pM $1,25(OH)_2D_3$ (n = 3), and mean ± range for 26 and 1,300 pM steroid (n = 2). Lower case letters (a, b) indicate significant differences ($P < 0.05$) between specific activities obtained for different hormone concentrations.

These findings closely parallel the results for dose-response studies on phosphate transport [Nemere, 1996b].

Similar experiments performed for steroid-mediated PKA activation demonstrated increasing stimulation to an optimum at 650 pM $1,25(OH)_2D_3$ (Fig. 7), which corresponds to the optimum for calcium transport [Nemere et al., 1984; Yoshimoto and Norman, 1986]. Thus, the age-related decline in calcium transport is, in part, secondary to the decline in phosphate uptake [Zhao and Nemere, 2002], and presumably transport.

DISCUSSION

In the current work, age-associated changes in rapid, $1,25(OH)_2D_3$-induced calcium transport were studied in female chickens ranging in age from pullets to egg-laying hens. Both the immature and mature ages have a high demand for calcium, but for different reasons, that is, young birds require substantial calcium for bone formation during growth, while adult hens require calcium for shell formation. The results indicate that the rapid, $1,25(OH)_2D_3$-enhanced intestinal calcium transport is greatest in young birds (threefold increase over controls), diminishes to a twofold increase over controls at 14–28 weeks, and 1.5-fold in year-old hens. These observations parallel those in male birds [Larsson and Nemere, 2003]. Thus, the physiological role of this transport mechanism appears to support the demands of bone growth. The similarity in levels of hormone-stimulated calcium transport between 14- and 28-week-old females, as well as males [Larsson and Nemere, 2003], argues against an effect of altered dietary calcium levels on $1,25(OH)_2D_3$ responsiveness. Although vascular calcium levels have been shown to modulate $1,25(OH)_2D_3$ responsiveness [de Boland and Norman, 1990b], the effects are rapidly reversible [Yosimoto et al., 1986; de Boland and Norman, 1990b], and thus, in the current investigation, eliminated by the basal perfusion period.

In attempting to define the mechanism of this age-related change, we found alterations in several interrelated factors, namely binding parameters and signal transduction. Saturation

binding studies revealed an age-dependent decrease in the affinity of the 1,25D$_3$-MARRS bp for ligand, an observation paralleled in male chickens [Larsson and Nemere, 2003]. In addition, in female birds an apparent decrease in positive cooperativity of binding occurred. The apparent age-related increase in B_{max} for the binding of 1,25(OH)$_2$D$_3$ to isolated BLM may be due to lower affinity, higher capacity components [Larsson and Nemere, 2003]. These combined observations indicate a progressive decrease in the ability of the duodenum to respond rapidly to 1,25(OH)$_2$D$_3$.

Partially offsetting the impaired responsiveness of the duodenum might be increased serum levels of 1,25(OH)$_2$D$_3$. Circulating 1,25(OH)$_2$D$_3$ levels in female chicken have been reported [Sedrani, 1984] to be 0.17 ± 0.04 ,0.13 ± 0.02, and 0.34 ± 0.03 nM for 8-, 15-, and 23-week-old chickens, respectively. These values are somewhat lower than the affinities determined for the 1,25D$_3$-MARRS bp, but still within the effective range.

The ability of 1,25(OH)$_2$D$_3$ to activate PKC has been related to the presence of the 1,25D$_3$-MARRS bp by antibody inhibition studies both in intact cells [Nemere et al., 2000], and in membrane preparations lacking the VDR [Nemere et al., 1998]. Thus, declining hormonal stimulation of PKC activity observed in the present study can be related to age-associated binding parameters of the 1,25D$_3$-MARRS bp. We and others [Balogh et al., 1997] have also found an increase in basal PKC activity with age, although the significance of this observation is unclear, especially since basal PKC activity declined with age in male chickens [Larsson and Nemere, 2003]. The dose-response curves for PKC activation in the current work, and stimulation of phosphate transport [Nemere, 1996b] are quite similar. In addition, phorbol ester, a PKC stimulator, has been shown to enhance phosphate transport in perfused duodena [Zhao and Nemere, 2002], whereas forskolin, a PKA stimulator, did not [Zhao and Nemere, 2002]. Thus, diminished phosphate transport may be the primary age-related defect, with calcium movement secondarily affected. Studies are under way to test the effect of age on phosphate transport.

Basal PKA activity remained constant during the developmental phases in female chickens (as well as in males [Larsson and Nemere, 2003]), while the magnitude of 1,25(OH)$_2$D$_3$ stimulation increased with age (unlike studies with rats [Massheimer et al., 1995] and male chickens [Larsson and Nemere, 2003]). In young chicks, steroid activation of PKA was maximal at 650- to 1,300-pM 1,25(OH)$_2$D$_3$. Calcium transport in the perfused duodenal loop has been found to be optimal at 650-pM 1,25(OH)$_2$D$_3$ [Nemere et al., 1984; Yoshimoto and Norman, 1986], but declines at greater than 1-nM hormone [Yoshimoto and Norman, 1986]. In the current work, optimal stimulation of PKA occurred in enterocytes from 58-week birds, while calcium transport declined in this age group. Possible explanations include a decrease in PKA substrate with age to account for an absence of corresponding stimulated transport, and/or the potential involvement of a receptor system different than the 1,25D$_3$-MARRS bp to account for age-related increases in stimulation of PKA.

Our findings have confirmed the report [Wu et al., 1993] that basal intestinal calcium transport in immature (age 11 weeks), point of laying (age 17 weeks), and egg-laying birds (age 25 weeks) has been reported to increase twofold between point of laying and egg-laying birds, while the basal calcium transport was not significantly changed when comparing immature and point of laying. Thus, another interpretation of the combined data is that if calcium transport in egg-laying birds is at, or close to, maximum, a further stimulation with 1,25(OH)$_2$D$_3$ will not have as a great effect on the transport as a stimulation in younger birds having lower basal calcium absorption. However, saturation analyses of the nVDR indicate a significant increase in the number of receptors after 7 weeks of age, suggesting that enhanced basal transport of calcium in vivo is mediated by transcriptional regulation of the transcellular pathway components. A portion of this regulation may be through expression of a adenylate cyclase. Vitamin D deficiency has been reported to diminish adenylate cyclase activity [Corradino, 1974] and signaling [Nemere and Campbell, 2000]. In the present work, we observed an increased ability of 1,25(OH)$_2$D$_3$ to stimulate PKA activity, which depends on the presence of adenylate cyclase-synthesised cAMP. While this may be in part regulated by the nVDR, the relationship is not a simple linear one, that is, nVDR levels increased between 7 and 14 weeks, while hormonal stimulation of PKA activity increased continually with age. In

this context, it should be noted that the levels of nVDR are not always directly proportional to either calbindin [Fleet et al., 2002; Song et al., 2003] or calcium transport [Wu et al., 1994; Fleet et al., 2002; Song et al., 2003], further complicating mechanistic investigations.

Finally, the distinct differences in binding parameters between the $1,25D_3$-MARRS bp and nVDR, and relationship to rapid calcium transport during growth versus eggshell formation, suggest that the two proteins are separate moieties. These results run counter to the argument that the nVDR must be responsible for all membrane-initiated phenomena [Erben et al., 2002].

In conclusion, our results show that young growing birds having high calcium requirements respond more robustly to $1,25(OH)_2D_3$ stimulation than older birds. The decreased responsiveness can be explained both by a decreased affinity of $1,25D_3$-MARRS bp for $1,25(OH)_2D_3$, and decreased PKC activity. The onset of egg-laying and egg-laying itself does not seem to have any effect on the rapid $1,25(OH)_2D_3$ induced intestinal calcium uptake, even though the calcium needs are again high. Further, this study gives proof for the existence of a membrane-associated $1,25(OH)_2D_3$ receptor having different physiochemical properties from the classical nVDR.

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