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Parathyroid Hormone Stimulation of 1,25-Dihydroxyvitamin D_3 Production in Antiestrogen-treated Japanese Quail

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SUMMARY

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In vivo administration of estradiol benzoate (E_2B) or parathyroid hormone (PTH) stimulates *in vitro* renal conversion of 25-hydroxyvitamin D_3 [25-(OH)D_3] to 1,25-dihydroxyvitamin D_3 [1,25-$(OH)_2D_3$] in birds. In the present investigation, the effect of PTH on 1,25-$(OH)_2D_3$ production was studied in female Japanese quail pretreated with the antiestrogen, tamoxifen citrate (30 mg/kg) daily for 7 days. PTH was injected at two different doses (92 and 275 U.S.P. units/kg) every 8 hours during the last two days of tamoxifen treatment. E_2B (0.1 and 1.0 mg/kg) was injected 24 hours before sacrifice. Control groups received vehicle only. All injections were given intramuscularly. Kidney homogenates were prepared and incubated with tritiated 25-(OH)D_3. E_2B-induced stimulation of 1,25-$(OH)_2D_3$ production was completely blocked by prior treatment with tamoxifen, but PTH-induced stimulation of 1,25-$(OH)_2D_3$ production was unaffected by antiestrogen treatment. When PTH and E_2B were injected together, no additive effects on either 1,25-$(OH)_2D_3$ production or hypercalcemia were observed. The data indicate that estrogen receptors are not an essential link in the chain of events mediating the PTH-induced stimulation of 1,25-$(OH)_2D_3$ production.

INTRODUCTION

It is now firmly established that vitamin D_3 is metabolized in the liver and subsequently in the kidney to 1,25-dihydroxyvitamin D_3[1,25-$(OH)_2D_3$][1], which is the biologically active form of the vitamin (1-3) and has been designated a hormone (4). The kidney and possibly other tissues also produce 24,25-$(OH)_2D_3$, the exact biological role of which is yet unknown (5, 6). Among the many factors, both dietary and hormonal, that are known to influence the biosynthesis of 1,25-$(OH)_2D_3$ in the kidney, calcium and vitamin D (7, 8), phosphate (9, 10), and parathyroid hormone (10-12) have been studied more extensively, although the direct regulatory roles of parathyroid hormone (13-15) and phosphate (16, 17) are disputed. Recently, we and others have reported that administration of estradiol *in vivo* markedly stimulates *in vitro* renal synthesis of 1,25-$(OH)_2D_3$ in Japanese quail (18-21) and bullfrogs (22). We have also reported that estradiol appears to stimulate *in vivo* 1,25-$(OH)_2D_3$ production in rats (23). Since parathyroid hormone (PTH) is

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[1] The abbreviations used are: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 25-(OH)D_3, 25-hydroxyvitamin D_3; PTH, parathyroid hormone; E_2B, estradiol benzoate; TAM, tamoxifen citrate.

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known to stimulate 1,25-$(OH)_2D_3$ production (11, 12), whereas antiestrogen treatment (24) and ovariectomy (25) inhibit production, the present investigation was designed to study the interrelationships between PTH- and estradiol-induced stimulation of 1,25-$(OH)_2D_3$ production in antiestrogen (tamoxifen citrate)-treated female Japanese quail. Vitamin D metabolism was monitored *in vitro* using kidney homogenates and tritiated 25-$(OH)D_3$. A preliminary report of this work has appeared elsewhere (26).

METHODS

Birds. Four-week-old immature female Japanese quail (*Coturnix coturnix japonica*) were used. They were maintained and fed a normal diet (2.3 to 3.3% Ca and 0.8% P) as described elsewhere (19).

Hormones and chemicals. The antiestrogen, tamoxifen citrate (TAM) (kindly supplied by the Stuart Pharmaceutical Division of ICI United States, Wilmington, Delaware), was injected daily (30 mg/kg) for 7 days. Parathyroid injection U.S.P. (a gift from Eli Lilly and Co., Indianapolis) was injected at two different dose levels (92 and 275 U.S.P. units/kg) every 8 hours for 52 hours. Estradiol benzoate, U.S.P. (E_2B) was dissolved in 95% ethanol and injected at two dose levels (0.1 and 1.0 mg/kg) 24 hours before sacrifice. Control groups received an equal volume (1 mg/kg) of vehicle only. All injections were given intramuscularly. Each group contained 5 to 11 birds.

25-[26,27^{3}H]-$(OH)D_3$ (Amersham Searle, Chicago, Illinois, sp. act. 11.3 Ci/mmol) was used as a substrate. The substrate concentration in the incubation mixture was controlled at 0.2 μM following the addition of unlabeled 25-$(OH)D_3$ (kindly donated by Hoffmann-LaRoche, Nutley, N.J.) to give a final specific activity of 1.13 Ci/mmol.

Incubation of kidney homogenates and separation of metabolites. The preparation of kidney homogenates, the incubation with tritiated 25-$(OH)D_3$, and the subsequent extraction and separation of the metabolites were performed according to the method described by Kenny (27), as modified by Baksi and Kenny (24). The metabolite production was expressed as picomoles/min/g kidney.

Plasma calcium. Heparinized plasma was analyzed for calcium by atomic absorption spectrophotometry (Perkin-Elmer, Model 303).

Identification of metabolites. In addition to their chromatographic behavior on Sephadex LH-20 columns, the suspected metabolites were subjected to periodate oxidation (28) and Celite column chromatography as described by Haussler and Rasmussen (29). In our hands, Celite chromatography (using a 1 × 36 cm column of Celite equilibriated with 45% water in ethanol, eluting with 10% ethylacetate in hexanes, and collecting 5-ml fractions) is capable of separating 25,26-$(OH)_2D_3$ from 1,25-$(OH)_2D_3$ as described in the RESULTS section.

Statistics. Where appropriate the data are presented as mean values accompanied by the standard errors of the mean. Comparisons were made using Student's *t*-test.

RESULTS

Effect of PTH and E_2B on vitamin D hydroxylases and plasma calcium in normal and antiestrogen-treated Japanese quail

Lower doses of hormones. Both PTH (92 U.S.P. units/kg) and E_2B (0.1 mg/kg) significantly increased 1,25-$(OH)_2D_3$ production in normal birds at the lower dose levels (Fig. 1, top panel), but the reciprocal suppression of 24,25-$(OH)_2D_3$ was not evident with either hormone (Fig. 1, middle panel). In fact, PTH significantly increased 24,25-$(OH)_2D_3$ production at this dose. Injection of TAM (3 mg/kg for 7 days) alone decreased the activity of the 1-hydroxylase and increased that of the 24-hydroxylase. When the same doses of the hormones were administered to antiestrogen-treated birds, two different types of responses were observed. The same dose of E_2B failed to affect either the 1- or the 24-hydroxylase activities relative to the TAM-treated birds. PTH, on the other hand, was still effective in the presence of TAM; the production of 1,25-$(OH)_2D_3$ was stimulated and that of 24,25-$(OH)_2D_3$ was diminished. When PTH and E_2B were injected together in the absence of TAM, the response in

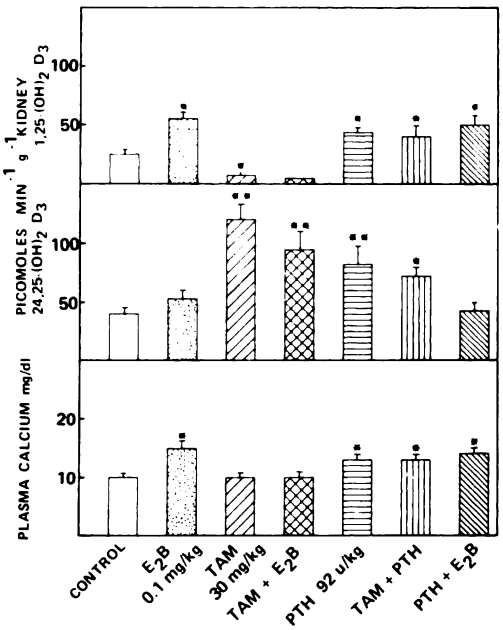

FIG. 1. *Vitamin D_3 metabolite production* in vitro *by kidney homogenates at low hormone dosage*

Kidney homogenates were prepared from immature female Japanese quail treated either with 95% ethanol, tamoxifen citrate (TAM) (30 mg/kg), TAM (30 mg/kg) and estradiol benzoate (E_2B; 0.1 mg/kg), PTH (92 U.S.P. units/kg), TAM and E_2B, and PTH and E_2B. TAM was given daily for 7 days; PTH every 8 hours for 52 hours; and E_2B as a single dose 24 hours before sacrifice. All injections were given i.m. Each group contained 5 to 11 birds. The kidney homogenates were incubated with 25-[26,27^{3}H]-$(OH)D_3$. The metabolites were extracted and separated using Sephadex LH-20 columns (0.7 × 18 cm) and chloroform/hexanes (65:35) solvent system. Fifty 125-drop fractions were collected and each fraction was counted by liquid scintillation. TAM either alone or with E_2B significantly ($p < 0.05$) reduced 1,25-$(OH)_2D_3$ production but PTH injection in untreated and TAM-treated groups significantly ($p < 0.05$) increased 1,25-$(OH)_2D_3$ production. 1,25-$(OH)_2D_3$ production in PTH + E_2B group was not significantly different from the groups receiving either E_2B or PTH alone. Production of 24,25-$(OH)_2D_3$ was significantly ($p < 0.01$) increased in the TAM-treated as well as in the PTH-treated group. Plasma calcium levels increased significantly ($p < 0.05$) in all PTH-treated groups and in the E_2B-injected group without TAM. Vertical brackets represent S.E.; $^*p < 0.05$; $^{**}p < 0.01$.

1,25-$(OH)_2D_3$ synthesis to the combination was no greater than the responses to either hormone when given alone. Whereas the hypercalcemic response to E_2B was completely blocked by TAM pretreatment, the plasma calcium response to PTH was unaffected by the antiestrogen (Fig. 1, bottom panel). The hypercalcemic response to the combination of hormones was not significantly greater than that to either hormone when given separately.

Higher doses of hormones. The results with higher doses of hormones (PTH, 275 U.S.P. units/kg; E_2B, 1.0 mg/kg) were qualitatively similar to those observed with the lower doses of the hormones with few exceptions (Fig. 2). Whereas the lower dose of PTH *stimulated* 24,25-$(OH)_2D_3$ production, the higher dose completely *suppressed* synthesis of this metabolite (Fig. 2, middle panel). Although TAM did blunt the 1,25-$(OH)_2D_3$ response to the higher dose of PTH, its alteration of this response was decidely different from the complete blockade of the response to E_2B.

The hypercalcemic response to the higher dose of PTH was again unaffected by antiestrogen pretreatment in contrast to the blockade of the hypercalcemic response to E_2B (Fig. 2, bottom panel).

Identification of Vitamin D_3 Metabolites

Periodate oxidation. Periodate oxidation of suspected 1,25-$(OH)_2D_3$ (Fig. 3, upper panel) and 24,25-$(OH)_2D_3$ (Fig. 3, lower panel) metabolites shows that incubation for 3 hours with 5% aqueous sodium periodate ($NaIO_4$) destroyed almost 40% of the 24,25-$(OH)_2D_3$, whereas similar treatment destroyed only 5% of the 1,25-$(OH)_2D_3$.

Celite chromatography. Celite column chromatography of the suspected 1,25-$(OH)_2D_3$ resulted in a single peak at fraction 25 (Fig. 4). These observations indicate that 25,26-$(OH)_2D_3$ is not a major contaminant of the 1,25-$(OH)_2D_3$ peak; Celite chromatography under these conditions separates the two metabolites as demonstrated below.

Separation of 1,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$. The following experiment was done in order to demonstrate that: (1) Celite chromatography, under the conditions used, adequately separates 1,25-$(OH)_2D_3$, 24,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$; and (2) Sephadex LH-20 chromatography using a 1 × 50 cm column and a hexanes/chloroform/methanol (9:1:1) solvent system also

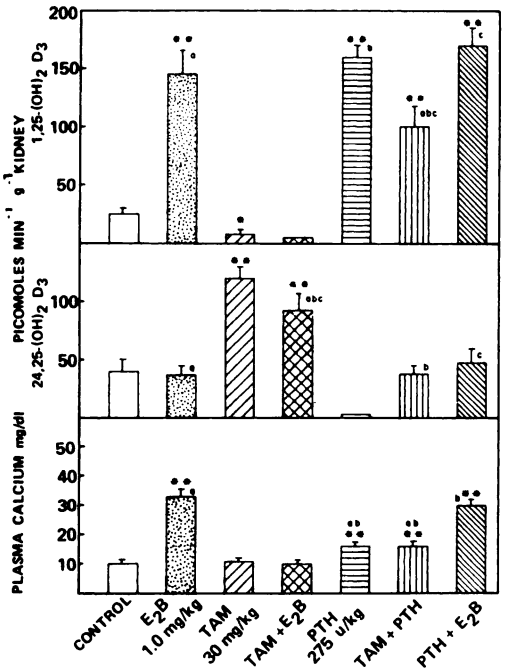

FIG. 2. *Vitamin D_3 metabolite production* in vitro *by kidney homogenates at high hormone dosage*

The conditions were identical to those in Figure 1 except that higher doses of PTH (275 U.S.P./kg) and E_2B (1.0 mg/kg) were used. The pattern of 1,25-$(OH)_2D_3$ and 24,25-$(OH)_2D_3$ production was similar to that seen with the lower doses of PTH and E_2, except that higher dose of PTH suppressed 24,25-$(OH)_2D_3$ production to nondetectable levels in normal birds and significantly ($p < 0.05$) reduced the levels in the TAM-treated group. Groups with similar letters (a, b, or c) are significantly different ($p < 0.05$) from each other.

separates the three metabolites. The 25,26-$(OH)_2D_3$ metabolite was generated biosynthetically by a modification of the method of Tanaka *et al.* (30), who found that a single injection of vitamin D_3 into vitamin D-deficient chickens stimulated 26-hydroxylase activity within 48 hours. The major modification introduced in the experiment to be described was the use of Japanese quail instead of chickens.

Four-week old female Japanese quail were placed on a vitamin D-deficient diet (Rachitogenic Test Diet, cat. no. 170660, Teklad Test Diets, Madison, WI) for 4 weeks. At 8 weeks of age, two quail were injected intramuscularly with 6.5 nmol of cystalline vitamin D_3 (Sigma Chemical Co., St. Louis, MO) dissolved in 95% ethanol; 2 control quail were injected with an equal volume (100 μl) of 95% ethanol. Forty-eight hours after the administration of the vitamin D_3 or ethanol, the kidneys were removed, homogenized, and incubated with 10 nM 25-[26,27-^{3}H](OH)D_3.

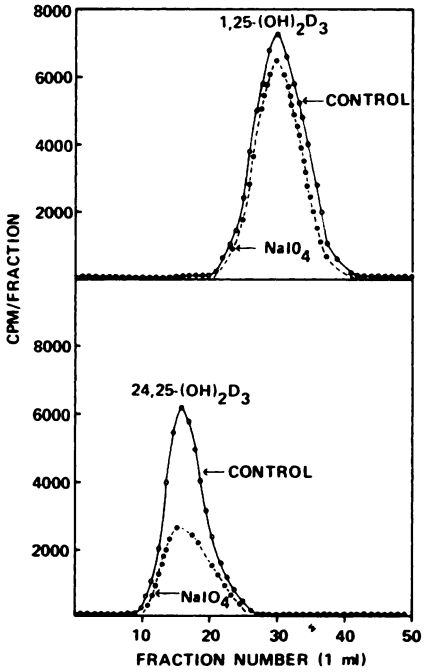

FIG. 3. *Periodate treatment of suspected 1,25-$(OH)_2D_3$ (upper panel) and 24,25-$(OH)_2D_3$ (lower panel) followed by Sephadex LH-20 (chloroform/hexanes; 65:35) chromatography*

Only a small amount (5%) of the 1,25-$(OH)_2D_3$ but 40% of the 24,25-$(OH)_2D_3$ disappeared after periodate treatment for 4 hours.

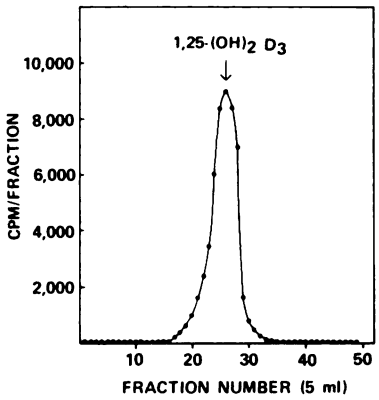

FIG. 4. *Celite column (1 × 36 cm) chromatography of suspected 1,25-$(OH)_2D_3$ with ethyl acetate/hexanes (10:90)*

A single peak appeared at fraction 25.

The homogenates were extracted and subjected initially to chromatography on a small (0.7 × 18 cm) column of Sephadex LH-20 using the chloroform/hexanes (65:35) solvent system as described by Kenny (27). A small aliquot of each fraction was counted to locate the peaks; the results are presented in Figures 5a (ethanol-treated)

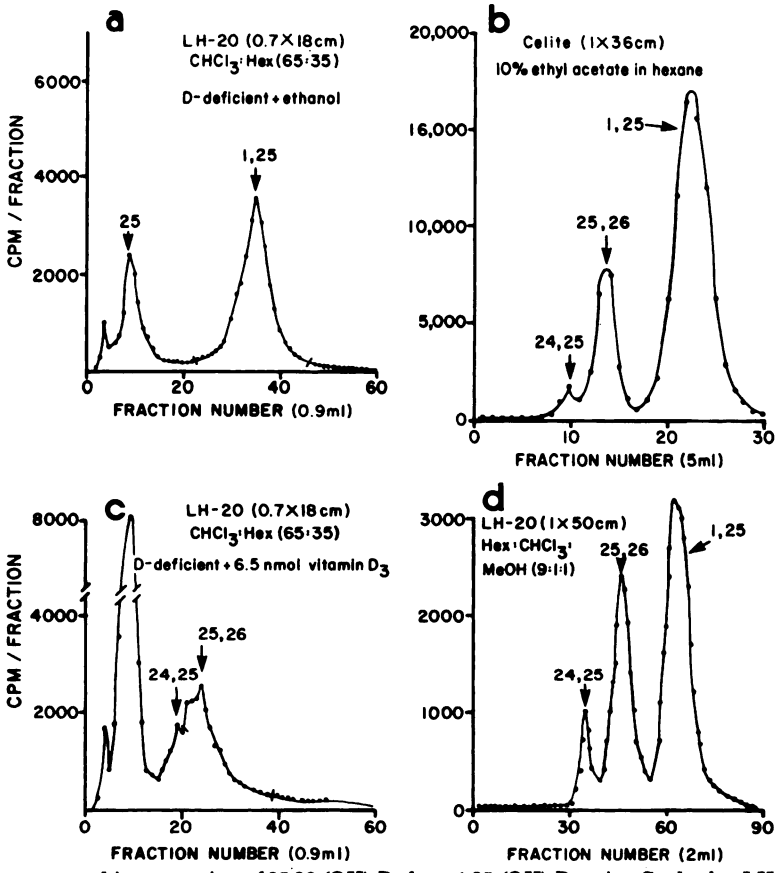

FIG. 5. *Chromatographic separation of 25,26-(OH)$_2$D$_3$ from 1,25-(OH)$_2$D$_3$ using Sephadex LH-20 and Celite columns*

Each of the two metabolites was biosynthesized from 25-[26,27-^{3}H](OH)D$_3$ using appropriate conditions as described in the RESULTS section: (1) 1,25-(OH)$_2$D$_3$ was generated from homogenates of kidneys removed from 8-week old Japanese quail which had been placed on a vitamin D-deficient diet at 4 weeks of age and injected i.m. with 100 μl of 95% ethanol 48 hours before removal of the kidneys; (2) 25,26-(OH)$_2$D$_3$ was synthesized from kidneys removed from similarly treated quail except that the 100 μl of 95% ethanol contained 6.5 nmol of vitamin D$_3$. The *left-hand* panels (a) and (c) represent the chromatographic profiles of the metabolite products of the ethanol-treated [1,25-(OH)$_2$D$_3$] and vitamin D$_3$-treated [25,26-(OH)$_2$D$_3$] quail, respectively, using small (0.7 × 18 cm) Sephadex LH-20 columns and the widely used chloroform/hexanes (65:35) solvent system. Whereas 1,25-(OH)$_2$D$_3$ peaked at fraction 35, the 25,26-(O)$_2$D$_3$ peaked earlier, at fraction 24, indicating that even this system is capable of separating these metabolites. The peak at fraction 19 in the bottom left-hand panel (c) is probably 24,25-(OH)$_2$D$_3$. The *right-hand* panels (b) and (d) represent the chromatographic profiles using Celite (1 × 36 cm with 10% ethyl acetate in hexanes) and Sephadex LH-20 (1 × 50 cm with 9:1:1 hexanes/chloroform/methanol), respectively, of a *mixture* of equal aliquots of combined fractions 28–44 [1,25-(OH)$_2$D$_3$] from the ethanol-treated birds (panel a) and of combined fractions 21–31 [25,26-(OH)$_2$D$_3$ plus 24,25-(OH)$_2$D$_3$ contaminant] from the vitamin D$_3$-treated birds (panel c). Both columns exhibited excellent separation of the 1,25-(OH)$_2$D$_3$ and 25,26-(OH)$_2$D$_3$ metabolites from each other and also from the 24,25-(OH)$_2$D$_3$ contaminant. The performance of the 1 × 50 cm Sephadex LH-20 column using hexanes/chloroform/methanol (9:1:1) was particularly impressive in view of its convenience as compared to Celite chromatographic method.

and 5c (vitamin D_3-treated), where the putative 1-25-$(OH)_2D_3$ and 26,26-$(OH)_2D_3$ peaks were found at fractions 35 (Fig. 5a) and 24 (Fig. 5c) respectively. The fractions forming the 1,25-$(OH)_2D_3$ (fractions 28–44, Fig. 5a) and 25,26-$(OH)_2D_3$ (fractions 21–31, Fig. 5c) peaks were combined into a single mixture. Separate aliquots of this sample containing the mixture of the putative 1,25-$(OH)_2D_3$ and 25,26-$(OH)_2D_3$ metabolites were dried under a stream of nitrogen. One such aliquot was dissolved in 300 μl of 10% ethyl acetate in hexanes, which had been equilibrated with 45% water in ethanol, and subjected to chromatography using a Celite column (1 × 36 cm) and collecting thirty-five 5-ml fractions (29). The results, presented in Figure 5b, indicate the presence of two major peaks at fractions 14 and 22, which are presumed to be 25,26-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$, respectively. Another such aliquot was dissolved in 300 μl of hexanes/chloroform/methanol (9:1:1) and subjected to chromatography with this solvent system using a large (1 × 50 cm) Sephadex LH-20 column and collecting 80 2-ml fractions (30). The results, presented in Figure 5d, again indicate the presence of two major peaks (fractions 45 and 62) which are presumed to be 25,26-$(OH)_2D_3$ and 1,25-$(OH)_2D_3$ respectively. The less polar minor peaks (fraction 10, Fig. 5b and fraction 35, Fig. 5d) are assumed to be 24,25-$(OH)_2D_3$. The data presented in Figure 5b indicate that the Celite column used for identification of the putative 1,25-$(OH)_2D_3$ peak (Fig. 4) is indeed capable of separating 1,25-$(OH)_2D_3$ from 25,26-$(OH)_2D_3$. We were especially impressed with the resolving power of the 1 × 50 cm Sephadex LH-20 column using the hexanes/chloroform/methanol (9:1:1) solvent system (Fig. 5d), a technique which, in our hands, is much more convenient and rapid than Celite chromatography.

DISCUSSION

The present study indicates that estrogen receptors are not an essential link in the chain of events mediating the PTH-induced stimulation of renal 25-$(OH)D_3$-1-hydroxylase in immature Japanese quail. This conclusion is based on the finding that, whereas antiestrogen-treated Japanese quail failed to show any stimulation of the 1-hydroxylase enzyme when estradiol was injected, the response to PTH was qualitatively unaffected by the antagonist. Although PTH and estradiol, in their action on the renal 1-hydroxylase, do not appear to have identical mechanisms, it is possible that they have certain components of their pathways in common. What are some of the possible mechanisms by which each hormone stimulates renal 1-hydroxylase activity? Does each hormone act directly on the kidney? Do the two hormones act through a common pathway? Does one hormone stimulate the release of the other?

Neither PTH (31) nor estradiol (31, 32) appears to act directly on the kidney. Nevertheless receptors are present in the avian kidney for both PTH (33) and estradiol (34). In the case of PTH this is not surprising in view of its well-known effects on renal tubular reabsorption of phosphate and calcium; estradiol, on the other hand, has no known direct action on renal function.

Is it possible that the two hormones act through a common pathway? Both hormones are hypercalcemic in avian species (Figs. 1 and 2); perhaps stimulation of 1-hydroxylase activity is related to this common response. Other observations from our laboratory, however, militate against this specific mechanism. Whereas the hypercalcemic response to PTH occurs *before* the 1-hydroxylase response (35), the plasma calcium response to estradiol occurs *after* the enzyme response (28). Perhaps prolactin release is common to both pathways. Spanos *et al.* (36, 37) have reported that prolactin stimulates renal 1-hydroxylase activity in the chicken, an observation which has been confirmed in our laboratory in Japanese quail (22). Spanos *et al.* suggested that the response to estradiol might be mediated through release of prolactin. Furthermore, PTH has been reported to induce a rise in plasma prolactin in the human (38). An attempt to approach this problem in the Japanese quail by using the dopaminergic agonist, bromocriptine, which is known to inhibit prolactin release in mammals (39), was frustrated by interpretation

(Baksi and Kenny, unpublished observations). The relationship of dopaminergic stimulation to prolactin release is unclear in avian species; birds appear to have a prolactin releasing factor (40) rather than a prolactin inhibitory factor as is the case in mammals.

Is it likely that one hormone stimulates the release of the other, which in turn activates the renal 1-hydroxylase? Does estradiol stimulate PTH release or does PTH stimulate estrogen release? The fact that treatment with the antiestrogen, tamoxifen, does not block the renal 1-hydroxylase response to PTH (Figs. 1 and 2) indicates that the action of PTH on the renal 1-hydroxylase is not mediated by the release of estrogen. The alternative mechanism, that estrogen causes the release of PTH, which in turn activates the renal enzyme, is also unlikely. The definitive study, either supporting or refuting this hypothesis, has yet to be reported. Nevertheless, the fact that estradiol, at least pharmacologically, activates the renal enzyme within 4 hours (28), that is *before* the first effects of PTH (12 hours) can be elicited (35), needs to be reconciled with any hypothesis proposing that estrogen acts through the release of PTH. Further evidence against the concept that estradiol stimulates the release of PTH may be gleaned by an examination of the hypercalcemic responses to both hormones. The hypercalcemic response to PTH has a very rapid onset in Japanese quail (35, 41), reaching a peak between 20 and 60 minutes, several hours before the hypercalcemic response to estradiol first appears (28). If it is proposed that estradiol activates 1-hydroxylase by first releasing PTH, then the absence of an early hypercalcemic response to estradiol (28) from endogenous PTH release needs to be explained.

The failure to observe an additive effect on 1-hydroxylase activation following injection of the lower, submaximal doses of both hormones deserves comment. No simple explanation is forthcoming; interpretation is complicated by the differences in time-response relationships between estradiol (28) and PTH (35). It is possible that one or both of the agents have partial agonist properties such that, under certain circumstances of time- and dose-response conditions, antagonistic characteristics are exhibited. In addition, the inhibiting influence of 1,25-$(OH)_2D_3$ itself on 1-hydroxylase activity (32) may be modifying the expected additive response.

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