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HYDROXYCHOLECALCIFEROLS MODULATE PARATHYROID HORMONE AND CALCITONIN SENSITIVE ADENYLYL CYCLASE IN BONE AND KIDNEY OF RATS

A POSSIBLE PHYSIOLOGICAL ROLE FOR 24,25-DIHYDROXY VITAMIN D_3

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Abstract—In particulate fractions from rat bone cells, but not from kidney, 24,25-$(OH)_2$ D_3 inhibits in a dose dependent manner (1 nM and above) the parathyroid hormone (PTH)-activated adenylyl cyclase. In contrast, 24,25-$(OH)_2D_3$ enhances the calcitonin(CT) stimulated cyclase in bone, but attenuates the CT-induced cyclase response in kidney. In supranormal concentrations 1,25-$(OH)_2D_3$ is also able to reduce the PTH-stimulated adenylyl cyclase in bone. In comparison, neither vitamin D_3 metabolite interferes with stimulation of adenylyl cyclase from pituitary cell membranes by thyroliberin (TRH) or vasoactive intestinal polypeptide (VIP). These findings may have important therapeutical consequences in preventing excessive PTH action and bone demineralization.

Among the known vitamin D compounds 1,25 dihydroxycholecalciferol (vitamin D_3; 1,25-$(OH)_2$ D_3) is the most active form and is believed to harbour the main biological effect which is to stimulate intestinal calcium absorption [1–4]. The kidney is the principle organ where the dihydroxylated metabolites, 1,25-$(OH)_2$ D_3 or 24,25-$(OH)_2$ D_3 is formed [1–4]. The general view is held today that 24,25-$(OH)_2$ D_3 is formed in the kidney as a metabolic degradation product of 25-OH D_3 [5, 6]. We show in this study that 24,25-$(OH)_2$ D_3 is able to change the response of adenylyl cyclase to human PTH and human CT in bone and kidney preparations of the rat.

MATERIALS AND METHODS

Materials. Human parathyroid hormone (PTH, 1–84 amino acids, Peninsula Lab. Inc., San Carlos, CA), human calcitonin (CT, 1–32 amino acids, Ciba-Geigy, Basel, Switzerland), Gpp(NH)p forskolin, and other chemicals used in this study were from Sigma Chemical Company (St. Louis, MO).

Tissue preparation and vitamin D_3 preincubation condition. For these experiments we prepared homogenate fractions from calvaries, kidneys and the anterior pituitary gland from freshly killed 40-day-old male Wistar rats.

The animals were decapitated, exsanguinated, and the calvaries were dissected, freed of connective tissue, minced and rinsed in ice cold saline (0.15 M) under gentle stirring for 5 min. Subsequent homogenization on ice with Ultra Turrax in a 10 mM Tris–HCl buffer, pH 7.5 (containing 1 mM EDTA) was followed by filtration through a nylon mesh. The homogenate was diluted with the Tris–EDTA buffer containing 0.1% bovine serum albumin (BSA) to yield a total protein concentration (including osteocyte membranes) of 1 mg/ml and preincubated with either no addition (control), 25-OH vitamin D_3 (0.01–1 μM), 1,25-$(OH)_2$ vitamin D_3 (0.01–1 nM) or 24,25-$(OH)_2$ vitamin D_3 (1–100 nM) for 20 min at 30° [7]. The homogenate was then centrifuged (27,000 *g* for 30 min at 4°), and the pellet resuspended in buffer containing BSA. Kidney and pituitary homogenates and membrane fractions were prepared essentially in the same manner. Membranes were assayed for adenylyl cyclase activity as described below.

Adenylyl cyclase assay. In brief, the assay was carried out in a final volume of 50 μl and 1 mM ATP (including 2.5×10^{-6} cpm/tube of α-^{32}P-ATP), 40 μM GTP, 1 mM cAMP (with 7800 cpm/tube of ^{3}H-cyclic AMP), 1.4 mM EDTA, 0.1 mM EGTA, 2.8 mM Mg^{2+}, 0.15 mM Ca^{2+} and 25 mM Tris–HCl (pH 7.4) together with a regenerating system for ATP consisting of 20 mM creatine phosphate, 0.2 mg/ml of creatine kinase and 0.02 mg/ml of myokinase. PTH, CT, Gpp(NH)p (guanyl 5′-yl imidodiphosphate) or forskolin were added just prior to the final incubation which was carried out at 35° for 20 min. The reactions were stopped with 0.1 ml of a "stopping solution" containing 10 mM cAMP, 40 mM ATP and 1% sodium dodecyl sulfate followed by mixing and immediate cooling to 0°. The ^{32}P-cAMP formed and the ^{3}H-cAMP added to monitor recovery were isolated using combined Dowex and alumina oxide chromatography [8, 9]. The overall recovery was 65–85 per cent and the reaction blanks were 3–6 cpm per 1×10^6 cpm of labelled ATP added. The final imidazole eluate was collected in scintillation vials containing 5 ml of Instagel (Packard) and radioactivity counted for 10 min in a liquid scintillation counter. The ATP, GTP and Gpp(NH)p concentrations were estimated by ultraviolet spectrophotometry at 260 nM.

The results are given as pmoles cAMP produced

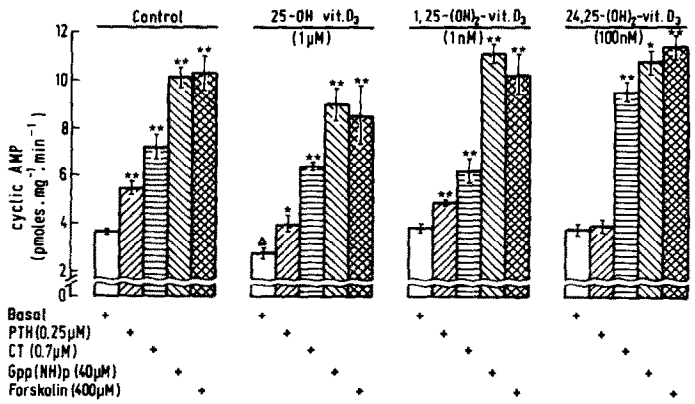

Fig. 1. The effect of different cholecalciferols (vit D_3 metabolites) on the PTH- and CT-sensitive adenylyl cyclase from calvaries of 40-day-old male Wistar rats. PTH and CT were used in concentrations that gave maximal stimulated adenylyl cyclase activity [8, 9]. The experiments were conducted as outlined in Materials and Methods. The vit D_3 metabolites were added to filtered crude homogenates in the concentrations indicated. Enzyme activity is expressed as pmoles cAMP per mg protein and min, and depicted as mean ± S.D. of triplicates. the experiment was performed twice. Statistical calculations were performed as described in Materials and Methods: * $P < 0.05$; ** $P < 0.01$ for comparison between basal and treatment groups. $^{\Delta}P < 0.01$ when basal control is compared to basal 25-OH vit D_3.

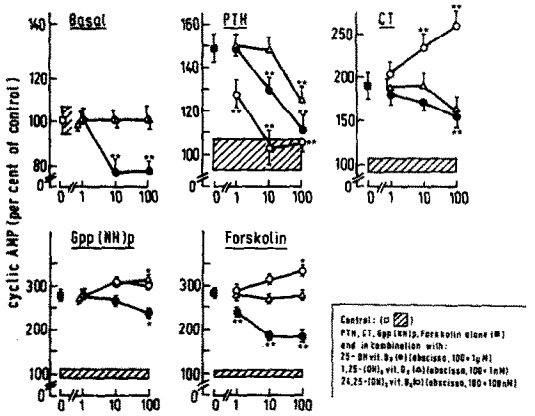

Fig. 2. The effects of vit D_3 metabolites on basal and stimulated adenylyl cyclase in rat calvaries. Abscissa indicates relative concentrations of the three vit D_3 analogues: Maximal value (100) represents for 25-OH vit D_3 1 μM (●); for 1,25-$(OH)_2$ vit D_3 1 nM (△) and for 24,25-$(OH)_2$ vit D_3 100 nM (○), respectively. The cholecalciferol metabolites were preincubated with the filtered crude bone cell homogenate, and the experimental conditions were otherwise as described in Materials and Methods. The concentrations of PTH, CT, Gpp(NH)p and forskolin were as indicated in Fig. 1 and they all elicited maximal enzyme activation [8, 9]. The enzyme activity which was measured as pmoles cAMP per mg membrane protein and min is given as per cent of non-stimulated controls. The results are depicted as mean ± S.D. of triplicates samples, and the experiment was carried out twice. * $P < 0.05$; ** $P < 0.01$.

per mg membrane protein per min and shown as mean values ± S.D. of triplicate determinations. All experiments were carried out twice.

We also used a particulate bound adenylyl cyclase from a clonal strain of rat pituitary cells which synthesises and secretes prolactin. These cells contain 1,25-$(OH)_2$ D_3 receptors, but are not target cells for PTH or CT.

Statistical calculations. The results are given as mean values of adenylyl cyclase activation and statistical significance assessed by the Wilcoxon nonparametric, two-sample test (two-tailed, $n_1 = n_2 = 6$).

RESULTS

Normal serum concentrations of 25-OH D_3, 1,25-$(OH)_2$ D_3 and 24,25-$(OH)_2$ D_3 in rats are on average 70 nM, 100 pM and 10 nM, respectively [7], and dose–response studies employing these concentrations were conducted (see Materials and Methods).

Figure 1 (control) describes the responsiveness of *the bone adenylyl cyclase system* to PTH, CT, Gpp(NH)p and forskolin using concentrations that gave maximal enzyme responses [8, 9]. In different experiments PTH stimulated the bone adenylyl cyclase activity 1.4- to 1.6-fold while CT caused an approximate 2-fold increase. The maximal stimulation of the adenylyl cyclase obtained by Gpp(NH)p and forskolin were similar and represented a 2.8-fold increase. When the bone cell homogenate fractions were preincubated with approximately 10 times the physiological concentrations of 25-(OH) vit D_3 (1 μM), 1,25-$(OH)_2$ vit D_3 (1 nM) or 24,25-$(OH)_2$ vit D_3 (100 nM), modifications of the hormone-, Gpp(NH)p- and forskolin-responses were observed. The 25-(OH) vit D_3 compound, but not the others induced a significant reduction ($P < 0.01$) in basal adenylyl cyclase activity. The 1,25-$(OH)_2$ vit D_3 had an inhibitory effect on the PTH- and CT-stimulated enzyme. In comparison, the effect of 24,25-$(OH)_2$ vit D_3 differed distinctly from the other two vit D_3 analogues since it blocked the PTH induced enzyme activation and potentiated the effect of CT. No biologically significant change in the cyclase activation caused by Gpp(NH)p and forskolin was observed using the dihydroxylated compounds.

The dose–response curves for the different vit D_3 analogues, Gpp(NH)p and forskolin on basal and hormone-stimulated adenylyl cyclase were then

examined. Figure 2 (basal) shows that only 25-OH vit D_3 is able to reduce significantly the basal adenylyl cyclase activity in bone cell homogenate fractions to about 80 per cent of control ($P < 0.01$). Moreover, 25-OH vit D_3 inhibits, but does not block the stimulating effect of PTH. In comparison 24,25-$(OH)_2$ vit D_3 reduces the PTH stimulated cyclase activity ($P < 0.01$) by about 50% already at 1 nM, and hormone stimulation is completely blocked at 10 nM (physiological concentration) and above. An attenuation of the PTH induced adenylyl cyclase in bone was also exerted by 1,25-$(OH)_2$ vit D_3 ($P < 0.01$) when tested at 1 nM, but not at lower and physiological concentrations. The two dihydroxylated compounds appeared to be equipotent on a molar basis when tested over the concentration range 0.1–100 nM (data not shown).

Dose dependent inhibitions, but no blockade of the CT sensitive cyclase in bone were also produced by 25-(OH) vit D_3 ($P < 0.01$) (Fig. 2, CT). In contrast, 24,25-$(OH)_2$ vit D_3 already at 10 nM ($P < 0.01$) enhanced the enzyme activity.

The guanyl nucleotide analogue Gpp(NH)p and forskolin stimulate the enzyme complex at two different sites distinct from the hormone receptor [10, 11]. The 25-(OH) vit D_3 metabolite interfered little with the action of Gpp(NH)p while a reduced forskolin stimulation was apparent already at 1 nM ($P < 0.01$). The 24,25-$(OH)_2$ vit D_3 enhanced moderately the forskolin-stimulated enzyme effect at the highest concentration (100 nM) ($P < 0.05$). The effect of 25-OH-vit D_3 on the hormone responsive cyclase could to a certain extent be explained by its interference with the two latter components of the enzyme complex. However, the effect of 24,25-$(OH)_2$ vit D_3 on the PTH and CT stimulated cyclase cannot be explained by the same mechanism since the stimulation caused by Gpp(NH)p and forskolin were unaltered or slightly enhanced.

The next question we asked was whether these modulatory effects of the hydroxylated vit D_3 metabolites were ubiquitous or subjected to tissue variations. Figure 3 depicts the impact of the vit D_3 analogues on the hormone-stimulated *kidney adenylyl cyclase*, which is another major target enzyme for PTH and CT actions. When the preincubation step was devoid of vit D_3 metabolites (Fig. 3, control), a distinct PTH- and CT-stimulated adenylyl cyclase was observed (1.7- and 1.4-fold increase, respectively). In comparison, the Gpp(NH)p and forskolin gave an approximate 4-fold stimulation. The basal adenylyl cyclase activity was only slightly reduced ($P < 0.05$) by 25-OH vit D_3 which also attenuated the CT- ($P < 0.05$), the Gpp(NH)p- and the forskolin-responses ($P < 0.01$) (Fig. 3). However, the PTH dependent kidney cyclase stimulation was not altered by any of the vit D_3 metabolites. Figure 4 shows the dose response effects of the different vit D_3 metabolites. The 25-OH vit D_3 caused again a small, but significant reduction ($P < 0.01$) in the basal enzyme activity and attenuated the stimulated enzyme ($P < 0.01$) except the response induced by PTH (Fig. 4). The dihydroxylated vit D_3 compounds showed no effect on the PTH induced cyclase activation, but attenuated the CT response ($P < 0.01$). Except for a moderate reduction ($P < 0.05$) in the CT induced cyclase response at 1 nM, 1,25-$(OH)_2$ vit D_3 did not alter the basal or stimulated hormone responses.

In crude membrane fractions from normal rat anterior pituitary glands or prolactin producing clonal rat strains (GH cells), the hypothalamic hormone thyroliberin (TRH) and vasoactive intestinal peptide (VIP) stimulate the adenylyl cyclase activity [8, 9]. Neither of the three vit D_3 metabolites was able to change these hormone-induced enzyme activations although 25-OH vit D_3 reduced the basal activity by some 20% ($P < 0.05$).

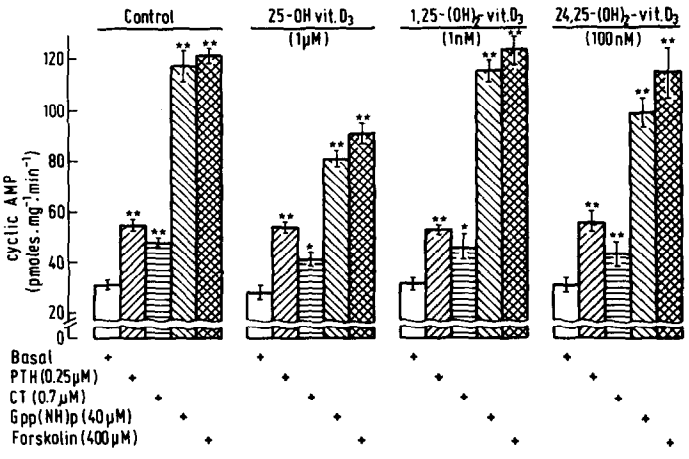

Fig. 3. The effect of vit D_3 metabolites on the PTH- and CT-sensitive adenylyl cyclase of kidneys from 40 days old male Wistar rats. PTH, CT, Gpp(NH)p and forskolin were used in concentrations that gave maximal activation of the adenylyl cyclase [8, 9]. The experimental protocol was as described in Materials and Methods. The three different vit D_3 metabolites were used in the concentrations indicated and preincubated with filtered crude kidney homogenates. The enzyme activity was measured as pmoles cAMP per mg protein and min, and the results are shown as mean ± S.D. of triplicates. The experiment was carried out twice. * $P < 0.05$; ** $P < 0.01$.

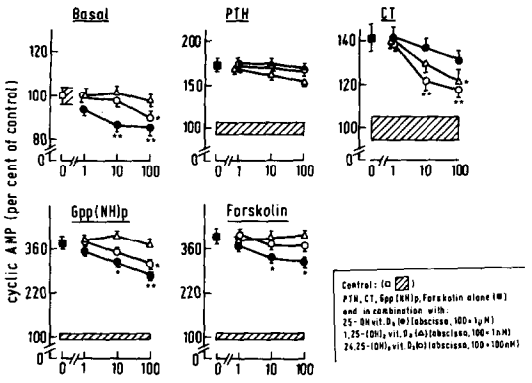

Fig. 4. The effects of vit D_3 metabolites on basal and hormone stimulated adenylyl cyclase in 40-day-old rat kidneys. Abscissa shows relative concentrations of the three vit D_3 analogues as explained in legend to Fig. 2. The cholecalciferol analogues were preincubated with the filtered crude kidney homogenates, and the experimental conditions were otherwise as described in Materials and Methods. The concentrations of PTH, CT, Gpp(NH)p and forskolin were as indicated in legend to Fig. 2 and gave maximal enzyme activation [8, 9]. The enzyme activity which was measured as picomoles cAMP per mg protein and min, was calculated as per cent of control (see legend to Fig. 2). The results are depicted as mean ± S.D. of triplicate samples, and the experiment was carried out twice. * $P < 0.05$; ** $P < 0.01$.

DISCUSSION

The 1,25-$(OH)_2$ vit D_3, but not the other vit D_3s, inhibits prolactin production in GH cells which have specific cytosolic receptors for 1,25-$(OH)_2$ vit D_3 [12].

The lack of acute effects by the three vit D_3 analogues on the hormone-sensitive adenylyl cyclase in pituitary cells therefore shows that the fat soluble sterols do not perturb the membrane in an unspecific manner. Not only are these effects organ specific, but within the same tissue, the 3 vit D_3 compounds affect the hormone sensitive adenylyl cyclases differently depending on which peptide hormone is added to the incubation mixture. Thus, in the bone cells, 24,25-$(OH)_2$ vit D_3 inhibits the PTH stimulated cyclase while the CT response is enhanced. In the kidney 24,25-$(OH)_2$ vit D_3 does not interfere with the PTH-stimulated adenylyl cyclase response, but attenuates to a small extent the stimulation caused by CT. The different actions of the vit D_3 analogues on the PTH and CT responsive cyclases in both tissues show that the sterols cannot interfere with binding between the hormones and their receptors. The mechanism for the acute effect of 24,25-$(OH)_2$ vit D_3 on the PTH-stimulated adenylyl cyclase in bone is unknown. The present data, however, rule out an unspecific perturbation of the function of the guanyl nucleotide binding protein or the catalytic site of the enzyme by this vit D_3 metabolite since the Gpp(NH)p- and forskolin-induced responses were not altered in the same manner. It seems also unlikely that these acute effects on the hormone dependent cyclase were conveyed via receptors for 24,25-$(OH)_2$ vit D_3 since similar results were obtained with a cytosol-free purified membrane preparation (data not shown). Furthermore, no receptor-protein has been found for 24,25-$(OH)_2$ D_3 in osteoblasts or in osteoclasts [13]. It is more reasonable to explain the results as a consequence of a 24,25-$(OH)_2$ vit D_3 dependent interference in the signal transduction system of the cell membrane between the hormone-bound receptor and the guanyl nucleotide binding component. The vit D_3 metabolites are seco steroids where one of the rings of the cyclopentano-perhydrophenanthrene structure characteristic of steroids has been broken, and they dissolve easily in the cell membrane structure. The different vit D_3 metabolites undergo a dynamic interchange between different conformations of the A ring [14] and this property together with hydroxylations at specific sites may explain the observed membrane effects. The present findings show that 24,25-$(OH)_2$ vit D_3 at physiological concentrations interferes in a specific manner with hormone action in bone cells: inhibiting the effect of PTH while enhancing the effect of CT on the membrane bound cyclase. Although, this study does not identify the vit D_3 sensitive cell types in bone and kidney, the results indicate that 24,25-$(OH)_2$ vit D_3 may play a hitherto unrecognized role in hormone-controlled bone remodelling. Studies to corroborate this hypothesis is presently being conducted in our laboratory.

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