Rapid accumulation of cyclic GMP near activated vitamin D receptors

(steroid receptor/signal transduction/guanylate cyclase/immunocytology)

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ABSTRACT The mechanisms of early calcitriol (1α,25-dihydroxycholecalciferol) effects, including its receptor activation process as well as its "nongenomic" effects, are poorly understood. Calcitriol causes a rapid accumulation of cGMP, dependent on the presence of normal vitamin D receptors (VDRs). We recently developed an immunocytology method based on rapid microwave fixation suitable to detect the locations of agonist-induced intracellular cGMP accumulation. With the same technique we found that calcitriol induces stepwise and rapid reorganization of VDRs. Here we used this technique to study the subcellular compartmentalization of cGMP accumulation after exposure of cells to various steroid-related agonists and to study the spatial relationship between cGMP accumulation and VDRs. Calcitriol (10 nM) within 15 sec caused clumping of VDRs and accumulation of cGMP around VDR clumps; thereafter (up to 5 min), the cGMP accumulation surrounded VDRs throughout their stepwise reorganization. In fibroblasts from subjects with mutations affecting VDR function, we found disruptions of the calcitriol-induced patterns of cGMP accumulation analogous to the disruptions of VDR reorganization. The colocalization of cGMP accumulation with reorganizing VDRs at early moments after calcitriol addition indicates transduction of the cGMP increase by VDRs inside the cell, rather than by components in the plasma membrane. Other steroid-related agonists caused compartmentalized and sequential changes in cGMP accumulation that seemed specific for each class of agonist. Our findings suggest that compartmentalized cGMP accumulation is an early and common step during activation of steroid-related receptors.

The early events during steroid and seco steroid hormone actions are associated with changes in the receptor, such as phosphorylation (1, 2), dimerization (3), dissociation from large complexes with 90-kDa heat shock protein (4), and changes in the interactions of receptor with other cell components [DNA (5), RNA (6), and other nuclear proteins (7)]. These changes may contribute to the receptors becoming competent for altering transcription of specific target genes.

Steroids also induce rapid cellular changes believed not to be directly related to receptor, such as change of intracellular cGMP (8), cAMP (9, 10), phospholipase A2 activity (11,12), pH (13), and membrane potential (14). The mechanism and the physiologic importance of rapid steroid effects are unknown. Recent evidence suggests that some rapid effects of calcitriol are mediated by the vitamin D receptor (VDR). The low half-maximum for calcitriol action, the calcitriol analog specificity, and defective response to calcitriol in cells with deficient or mutant VDRs were shown not only for rapid accumulation of cGMP (15,16) but also for rapid increase in intracellular calcium (9-11,17).

Since cGMP is potentially linked to some or all early effects of calcitriol, knowledge about the subcellular location of the cGMP accumulation (plasma membrane or intracellular) and its relation to VDRs could clarify the mechanisms of early steroid effects. An immunocytology method based on microwave energy fixation permits visualization of agonist-specific, dose-dependent, and time-dependent changes in cGMP compartmentalization (18) and in VDR organization (19). After live cells were loaded with fluorescent-labeled cGMP, fixation and immunostaining showed in vitro patterns of cGMP compartmentalization similar to those in vivo (18). Microwave fixation with different fixation methods has previously provided sufficient preservation of cell structure for light and electron microscopy (20).

MATERIALS AND METHODS

Reagents. 1α,25-Dihydroxyvitamin D3, 25-hydroxyvitamin D3, and 24,25-dihydroxyvitamin D3 were from M. Uskokovic (Hoffman-La Roche), 1β,25-dihydroxyvitamin D3 was from M. Holick and Rahul Ray (Boston University), and vitamin D3 was from Sigma. Dexamethasone, hydrocortisone, and progesterone were from Sigma. RU-486, deacylcorticazol, and 17α-hydroxyprogesterone were from S. Simons (National Institutes of Health). Retinoic acid, 3,5,3′-triiodo-1,3,5-thyronine, 17β-estradiol, and 17α-estradiol were from Sigma. Other reagents were from sources indicated previously (15, 18, 19).

Cell Sources. Normal dermal fibroblasts were from three subjects. Dermal fibroblasts from seven subjects with hereditary resistance to calcitriol (mutant fibroblasts) had VDR functional defects characterized previously (19, 21). We tested LLC-PK1 porcine kidney cells, CL9 rat liver cells, CV-1 monkey kidney fibroblasts (all from American Type Culture Collection), MCF-7 human breast cancer cells (gift of M. Lippman, Georgetown Univ., Washington), and UMR-106 rat osteosarcoma cells (gift of N. Patridge, Washington Univ., St. Louis).

Cell Culture, Fixation, and Immunostaining. Culture conditions, fixation, and immunostaining were as described (18,19). Briefly, cells were plated on LabTek culture slides coated with human fibronectin and cultured for 48 hr in medium without serum and phenol red, supplemented with ITS additive (insulin, transferrin, selenium, linoelic acid, bovine serum albumin). Calcitriol or other agonists were added at various concentrations and times in assay buffer (15) without the phosphodiesterase inhibitor isobutylmethylxanthine (IBMX), or in selected experiments in buffer with 0.5 mM IBMX. After calcitriol exposure, cells were fixed by 10-sec microwave exposure in an Amana model RCS 700 oven (18).

Abbreviations: VDR, vitamin D receptor; IBMX, isobutylmethylxanthine.

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Immunocytochemistry procedures were as described (18, 19). Briefly, cGMP was visualized with stepwise incubations in the following solutions (kits from Vector Laboratories): avidin blocking for 15 min, biotin blocking for 15 min, 4% horse serum for 30 min, mouse monoclonal antibody against cGMP (no. 4B6; diluted 1:800; gift of M. A. Kaliner, National Institutes of Health) in 4% human serum for 1 hr, biotinylated horse anti-mouse IgG for 30 min, avidin/biotinylated alkaline phosphatase for 30 min, levamisol/red substrate solution for 30 min. Double immunostaining included the following incubation steps: appropriate blocking solutions, 1 hr with rat monoclonal antibody against VDR (no. 9A7; diluted 1:1000; gift of J. W. Pike, Baylor College of Medicine, Houston), and rabbit polyclonal antibody against cGMP (18) (diluted 1:500; gift of K. J. Catt, National Institutes of Health), biotinylated second antibody to bind the 9A7 antibody, avidin/biotinylated phycoerythrin, then alkaline phosphatase-conjugated second antibody to bind rabbit cGMP antibody, and then levamisol/black substrate solution. Staining procedures were at room temperature, in a dark humidified box, with continuous tilting. All incubation steps were followed by three 5-min rinses with phosphate-buffered saline.

Pictures were taken with a Zeiss photomicroscope III equipped for epifluorescence analysis.

RESULTS

**cGMP Accumulation Pattern Before and After Calcitriol Addition to Cells.** Cells incubated in assay buffer without an added cGMP agonist showed very little cGMP signal near the plasma membrane and also very little diffusely in the cytoplasm.

CGRP accumulated as aggregates in the cytoplasm in normal fibroblasts within 15 sec (the earliest time tested) after addition of 0.1 μM calcitriol (Fig. 1). By 30 sec, the cGMP aggregates in 70% of cells were aligned in the cytoplasm along radially directed fibers. By 45 sec, the most intense accumulation of cGMP was along the nuclear envelope. At 1–3 min, cGMP aggregates were predominantly in the nucleus and in the nucleoli, and less cGMP accumulated in the cytoplasm than at 30 sec. The nuclear cGMP accumulation was maximal at 3 min, was stable at 3–5 min, was lower beyond 5 min, and was undetectable after 10 min. Threshold (0.1 nM) and maximal (1 μM) calcitriol doses caused the same sequential changes of cGMP accumulation pattern, but with higher doses the temporal sequence was more rapid and the cGMP signal was more intense. The threshold dose for cGMP accumulation was 1 μM for 1,25-dihydroxyvitamin D₃, 10 μM for vitamin D₃, 0.1 μM for 25-hydroxyvitamin D₃, and 10 nM for 24,25-dihydroxyvitamin D₃.

When IBMX was included in the incubation medium, cells without added cGMP agonists had mild cGMP accumulation near the plasma membrane; with IBMX in the medium, calcitriol caused a cGMP accumulation pattern similar to but with a more intense signal than that without IBMX in the medium.

Diverse cell lines (LLC-PK₁, CL9, MCF-7, UMR-106) responded to calcitriol with compartmentalized and sequential cGMP accumulation patterns indistinguishable from those in skin fibroblasts.

**Spatial Relations Between cGMP and VDR After Calcitriol Addition to Cells.** The time-dependent cGMP accumulation patterns after calcitriol addition were similar to the VDR reorganization patterns reported (19) with the following exception: the pattern of VDR reorganization was stable between 3 and 30 min, but cGMP accumulation had returned to baseline levels between 10 and 30 min. Double immunostaining of normal fibroblasts (data not shown) showed that after 15 sec with calcitriol (0.1 μM) cGMP accumulated immediately around the newly clumped VDRs, resulting in a small cGMP halo around the large VDR clumps. The portions of the VDR signal, which remained dispersed or refractory to reorganization, were not associated with cGMP halos. After 30 sec, cGMP aggregates were aligned and larger, with a bigger cGMP halo around VDR clumps. There were also VDR clumps apparently not aligned but surrounded by cGMP. Further from the clumped VDRs, a gradient of decreased cGMP accumulation was also evident. After 1 min with calcitriol, cGMP surrounded VDRs along the nuclear envelope and within the nucleus, and cytoplasmic cGMP was diminished and dispersed. After 3 min with calcitriol, the VDR-cGMP aggregates were almost exclusively intranuclear.

Double immunostaining for VDR and cGMP was also tested in UMR-106, LLC-PK₁, and CV-1 cells after calcitriol. The calcitriol binding capacities reported for these cells were as follows: UMR-106, 14,000 per cell (22); LLC-PK₁, 5400 per cell (23); CV-1, <500 per cell (24). We found substantially more VDR and also more calcitriol-induced cGMP in UMR-106 cells than in LLC-PK₁ cells or in dermal fibroblasts. The amount of VDR immunoreactivity was very low in CV-1 cells, but the amount of cGMP around the few VDR clumps was normal. The total amount of cGMP immunoreactivity

![Fig. 1. Compartmentalized cGMP accumulation patterns after addition of calcitriol (0.1 μM) to normal human dermal fibroblasts for the indicated times. (A) At 3 min, control (no calcitriol). (B) At 15 sec. (C) At 30 sec. (D) At 45 sec. (E) At 1 min. (F) At 3 min. (Bars = 10 μm.)](image-url)
after calcitriol in CV-1 cells was much less than in dermal fibroblasts.

**cGMP Accumulation Patterns Before and After Calcitriol Addition to Mutant Fibroblasts.** We also tested dermal fibroblasts from patients with different types of hereditary defects in VDR function (designated as “mutant” cells). We analyzed cGMP accumulation patterns alone and together with immunostaining of VDRs. cGMP response to calcitriol was subnormal or undetectable in all mutant cells (Table 1).

In fibroblasts with a point mutation in the DNA-binding region of the VDR, calcitriol caused a normal amount of cytoplasmic cGMP accumulation but with an unusual reticular pattern; however, cGMP accumulation above control was undetectable in the nucleus at all times including 3 min (Fig. 2; for comparison see Fig. 1F). In two mutant cell lines with undetectable or calcitriol-refractory VDR protein, calcitriol exposure did not induce cGMP accumulation. In one mutant line with a low number of VDRs, addition of calcitriol (0.1 nM to 1 μM) induced cGMP accumulation. The number of VDR–cGMP aggregates was very low, but the time-dependent changes in VDR patterns were otherwise normal. The abnormally low number of VDR–cGMP aggregates made them particularly suitable for photography of the colocalization (Fig. 3).

**Rapid cGMP Accumulation Patterns After Addition of Other Steroid-Related Agonists to Normal Fibroblasts.** We found in normal dermal fibroblasts broad similarities among the sequential changes in cGMP accumulation among all tested agonists at steroid-related receptors; initial cGMP accumulation was in the cytoplasm with accumulation at later times in the nucleus. cGMP always accumulated as discrete aggregates after addition of any steroid-related receptor agonist. We found that cGMP accumulation was highest after dexamethasone, somewhat lower after retinoic acid or calcitriol, even lower after triiodothyronine or dihydrotestosterone, very low after 17β-estradiol, and undetectable after progesterone.

The potency ratios of various glucocorticoid analogs to induce cGMP accumulation (deacylcortivasone > dexamethasone > hydrocortisone > 17α-hydroxyprogesterone >> progesterone) correlated with their affinity for binding to glucocorticoid receptors (26, 27). The glucocorticoid antagonist RU-486 (0.1 μM) induced cytoplasmic cGMP accumulation, but nuclear or perinuclear cGMP accumulation was not detectable at any time between 15 sec and 30 min. 17β-Estradiol (10 nM) and phenol red (15 mg/liter) induced nuclear cGMP accumulation, but 17α-estradiol (10 nM) did not.

The time course and the subcellular distribution of cGMP accumulation also revealed differences among the effect of various steroid-related receptor agonists on cGMP accumulation (Fig. 4). After dexamethasone (0.1 μM) cGMP accumulation was not detectable along the nuclear envelope, cytoplasmic cGMP decreased beyond 1 min, and cGMP did not decrease in the nucleus between 3 and 20 min. Dihydrotestosterone (0.1 μM) induced accumulation of cGMP aggregates within 15 sec, and the aggregates were closer to the periphery of the cytoplasm (just beneath the plasma membrane) than with other steroids. Cyclic GMP at its peak accumulation (1–2 min) was greater in the nucleus and nucleoli than in the cytoplasm. Beyond 2 min cGMP accumulation was diminished at all locations. 17β-Estradiol (0.1 μM) increased cGMP almost exclusively in the nucleus. Accumulation began as four to eight discrete aggregates within the nucleus, starting at 1 min and reaching maximal intensity at 5 min. After addition of triiodothyronine (0.1 μM), cGMP accumulated as aggregates in the cytoplasm without detectable alignment. Intranuclear cGMP aggregates were very small. cGMP accumulation was more prominent in the cytoplasm than in the nucleus, with little pattern change.

**Table 1. Patterns of cGMP accumulation after addition of calcitriol (10 μM or more) to fibroblasts from controls or subjects with hereditary defects in function of the VDR**

<table>
<thead>
<tr>
<th>Classification of VDR function</th>
<th>cGMP accumulation pattern after calcitriol</th>
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<tbody>
<tr>
<td>Normal (n = 3)</td>
<td>Aggregates in cytoplasm (15 sec), then radial alignment, mild perinuclear accumulation, then mainly in nucleus and including nucleoli</td>
</tr>
<tr>
<td>Undetectable calcitriol binding and markedly decreased VDR protein (n = 1)</td>
<td>Decreased numbers of cGMP aggregates but normal temporal and spatial sequence</td>
</tr>
<tr>
<td>Undetectable calcitriol binding and deficient or calcitriol-refractory VDR protein (n = 2)</td>
<td>No detectable cGMP accumulation even with 10 μM calcitriol</td>
</tr>
<tr>
<td>VCR cytosol-to-nucleus translocation defect (n = 2)</td>
<td>cGMP as aggregates only beyond 0.1 μM calcitriol; marked perinuclear but no intranuclear accumulation</td>
</tr>
<tr>
<td>Point mutation in DNA binding zinc finger domain of VDR (n = 2)</td>
<td>cGMP as aggregates only beyond 0.1 μM calcitriol; reticular cGMP accumulation in the Golgi region but no intranuclear accumulation</td>
</tr>
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VDR function was classified by prior studies of cell extracts’ interactions with [3H]-1α,25-dihydroxyvitamin D3 (21), immunocytochemistry of the VDR (19), and, for three of these mutant lines, identification of a homozygous point mutation in the VDR gene (19, 25). n, Number of subjects. Fibroblasts from each subject were analyzed in three or more similar experiments.

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**Fig. 2. Intracellular cGMP accumulation pattern 3 min after calcitriol (1 μM) (A) or control assay buffer (B) addition to human fibroblasts with mutation in the DNA binding region of VDR. (Bar = 10 μm.)**
between 3 and 10 min. Retinoic acid (0.1 μM) caused moderate cGMP accumulation in the cytoplasm at 15 sec mainly close to the nucleus, then very strikingly along the nuclear envelope with maximal accumulation at 2 min. Intranuclear cGMP aggregates were fewer and larger with retinoic acid than with other steroid-related agonists.

DISCUSSION

Several attempts have been made to identify the subcellular compartments in which the early effects of steroid hormone-related agonists are initiated. It has been suggested that rapid actions of steroids take place at the plasma membrane causing changes of its lipid composition (13, 28). Certain
rapid effects of calcitriol occurred in vitro with partially purified membranes (12). Some rapid calcitriol effects may not begin at the plasma membrane; calcitriol was shown to mobilize intracellular calcium (17) and to raise ionized calcium in isolated nuclei (29). Dependency of some rapid effects on the VDR (11, 15, 16) also suggests those effects are not initiated at the plasma membrane.

We have observed compartmentalized and time-dependent patterns of cGMP accumulation in the first minutes after addition of calcitriol and other steroid-related agonists to cultured cells. We previously found that other classes of agonists caused very different patterns of accumulation of cGMP or cAMP (18). In particular, our method was sufficiently sensitive and precise to detect cGMP accumulation along the plasma membrane after atrial natriuretic peptide exposure for 15 sec or more. However, we never detected cGMP accumulation along the plasma membrane after addition of calcitriol or an agonist at another steroid-related receptor.

The cGMP accumulation in the cytoplasm after addition of steroid-related agonists was always in a pattern of aggregates. This contrasted strikingly with the diffuse pattern of cGMP accumulation in the cytoplasm after addition of sodium nitroprusside or atrial natriuretic peptide (18). The spatial resolution of our analyses was not sufficient to establish whether the cGMP aggregates correspond to discrete organelles or simply to concentration gradients within a larger compartment.

Our observations establish a close spatial relationship between calcitriol-induced reorganization of VDRs and intracellular accumulation of cGMP: (i) cGMP aggregates showed similar patterns to those of VDR clumps (19) and were colocalized with VDR clumps (VDR clumping itself in either cytoplasm or nucleus seems to correlate temporally with receptor activation), (ii) the amount of cGMP accumulation after calcitriol exposure was proportional to the amount of VDR clumps in different cell lines, (iii) hereditary disruptions of VDR function caused appropriately coordinated disturbances in the patterns of VDR reorganization and of cGMP accumulation, and (iv) agonists for other steroid-related receptors also caused cGMP accumulation as aggregates, but the subcellular distributions differed in subtle ways from those observed after calcitriol.

Double immunostaining established that cGMP accumulated selectively around VDRs that had become clumped. Receptor aggregation was observed during glucocorticoid receptor activation (30); furthermore, oligomerization may be an obligatory step for activation of steroid-related receptors (3). Further studies will be required to establish whether cGMP accumulation precedes the clumping of VDRs, follows the clumping, or represents a parallel process. The functional consequences of these rapid VDR changes have not been established (19); clearly, cGMP could interact with diverse effector elements (31).

Since the presence of a phosphodiesterase inhibitor increased the cGMP signal after calcitriol but did not change its compartmentalization, it is likely that cGMP aggregates represent sites of increased synthesis and not sites of increased cGMP binding or of decreased cGMP degradation. It seems unlikely that the VDR has intrinsic guanylate cyclase activity (31, 32). Further studies will be required to determine the molecular mechanism through which the VDR increases cGMP synthesis.

We have used both immunocytochemistry and radioimmunoassay in dermal fibroblasts to study calcitriol effects on cGMP, and we found that the information with the two methods is complementary. Immunocytochemistry can provide information about the spatial distribution of cGMP; RIA can provide easy access to detection of quantitative changes. We found that immunocytochemistry is more sensitive to detect cGMP accumulation than RIA. It allowed detection of cGMP accumulation in normal cells 15 sec after addition of calcitriol and in some mutant cells after a high dose of calcitriol, although such accumulations had been undetectable by RIA (18). It is also possible that the pool of cGMP detected in cell extracts by RIA is partially different from the pool detected in intact cells by immunocytochemistry, because of the failure of RIA to detect a small component of GMP bound tightly to proteins (33).

The close spatial relationship between cGMP accumulation and VDR reorganization during the early minutes of calcitriol action shows that the rapid cGMP response to calcitriol is transduced by intracellular VDRs. Cyclic GMP accumulation in cytoplasm and/or nucleus could have an early role in activation of many or all steroid-receptor receptors and also could mediate some slower actions common to these receptors.