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Modulation by Epidermal Growth Factor of the Basal $1,25(OH)_2D_3$ Receptor Level and the Heterologous Up-Regulation of the $1,25(OH)_2D_3$ Receptor in Clonal Osteoblast-Like Cells

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Summary. The effects of epidermal growth factor (EGF) on basal 1,25-dihydroxyvitamin D_3 ($1,25\text{-}(OH)_2D_3$) receptor level and on parathyroid hormone (PTH)-induced $1,25\text{-}(OH)_2D_3$ receptor up-regulation were studied in the phenotypically osteoblastic cell line UMR 106. EGF in concentrations exceeding 0.1 ng/ml reduced the number of $1,25(OH)_2D_3$ binding sites without changing the binding affinity. Maximal reduction was 30% at about 1 ng/ml. This reduction was independent of a change in cAMP content. EGF dose-dependently attenuated both PTH-induced $1,25(OH)_2D_3$ receptor up-regulation and PTH-stimulated cAMP production, without an effect on the ED_{50} of the PTH effects. For both PTH responses the IC_{50} and the maximal effective dose were similar, 0.1 ng/ml and 1 ng/ml EGF, respectively. Reduction was first seen at 0.01 ng/ml EGF. At this concentration, EGF reduced PTH-stimulated $1,25\text{-}(OH)_2D_3$ receptor binding without an inhibition of the cAMP response. Time-course studies with 1 ng/ml EGF revealed that at 2 h preincubation EGF reduced the heterologous up-regulation by PTH, and maximal inhibition was seen after 4 h. In contrast, PTH-stimulated cAMP production was just significantly inhibited only after 6 h, with 60% inhibition after 24 h preincubation. The effects of prostaglandin E_2 and forskolin on both $1,25(OH)_2D_3$ binding and cAMP production were inhibited in a similar fashion. On the other hand, dibutyryl cAMP- and 3-isobutyl-1-methylxanthine-stimulated $1,25(OH)_2D_3$ binding were not affected by EGF. Taken together, our results demonstrate that EGF reduces both the basal number of $1,25(OH)_2D_3$ binding sites and the heterologous up-regulation of the $1,25(OH)_2D_3$ receptor. The current data suggest that EGF reduces heterologous up-regulation of the $1,25(OH)_2D_3$ receptor independent of as well as dependent on the cAMP messenger system. The EGF effect is not primarily located at the PTH receptor, at cAMP phosphodiesterase, or at protein kinase A level.

Key words: EGF-$1,25(OH)_2D_3$ binding – PTH – Osteoblast cell line.

$1,25$-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and parathyroid hormone (PTH) play an important role in calcium homeostasis. One of the target tissues for $1,25(OH)_2D_3$ and PTH is bone. For both hormones the receptors in bone are located on the osteoblast [1–4]. From *in vitro* as well as *in vivo* studies, evidence has been obtained indicating that $1,25(OH)_2D_3$ and PTH act in an interrelated fashion [5, 6]. Also at the level of the osteoblast, interactions between $1,25(OH)_2D_3$ and PTH have been reported. For instance, preincubation of osteoblast-like cells with $1,25(OH)_2D_3$ attenuates the stimulation of cAMP production by PTH [7–10]. Furthermore, we have recently reported that PTH and PTH-related protein cause heterologous up-regulation of the $1,25(OH)_2D_3$ receptor [11].

It has been shown that besides these two well-known calciotrophic hormones, growth factors and cytokines also affect bone cell metabolism. One of these polypeptide growth factors is epidermal growth factor (EGF) which has been shown to stimulate bone resorption *in vitro* [12, 13]. As for $1,25(OH)_2D_3$ and PTH, the receptor for EGF in bone is located on the osteoblast [14, 15]. EGF stimulates DNA and protein synthesis and prostaglandin production in osteoblasts of various origin [16–18] whereas collagen synthesis, hydroxyproline content, and alkaline phosphatase activity are reduced by EGF [16, 19]. Furthermore, EGF may modulate osteoblast responses to calciotrophic hormones. Recently, evidence has been obtained that EGF reduces the stimulation of cAMP production by PTH in the clonal osteoblast-like cells UMR 106 [20]. Also in several other cell types EGF has been found to modulate hormone responses [21–25].

To further understand the complex process of bone metabolism it is of considerable importance to study the interactions between the calciotrophic hormones and growth factors. Furthermore, imbalance of these interactions may be related to clinical disorders, e.g., humoral hypercalcemia of malignancy. In the present study we have examined the interactions between $1,25(OH)_2D_3$, PTH, and EGF in the phenotypically osteoblastic cell line UMR 106 [26]. First, we evaluated the effect of EGF on cellular $1,25(OH)_2D_3$ receptor levels. Second, we assessed whether the inhibitory effect of EGF on PTH-stimulated cAMP production is paralleled by an inhibition of a biological response to PTH, i.e., the up-regulation of the $1,25(OH)_2D_3$ receptor.

Materials and Methods

EGF, bPTH(1-34), prostaglandin E_2 (PGE_2), and dibutyryl cAMP (Bt_2cAMP) were obtained from Sigma, St. Louis, MO, USA. Forskolin was purchased from Calbiochem-Behring, USA, and 3-isobutyl-1-methylxanthine (IBMX) from Aldrich Chemie, Brussels, Belgium. [23,24-^{3}H]$1,25(OH)_2D_3$ (90 Ci/mmol) was obtained from Amersham International, England, and nonradioactive $1,25(OH)_2D_3$ was generously provided by LEO Pharmaceuticals, Denmark. Fetal calf serum (FCS), α-Minimal Essential Medium (α-MEM), penicillin, streptomycin, and glutamine were from Flow Laboratories (Irvine, Ayrshire, Scotland). All other reagents were of the best grade commercially available.

Culture and Treatment of the Cells

UMR 106 cells were seeded at 60,000 cells/cm^2 and cultured for 24

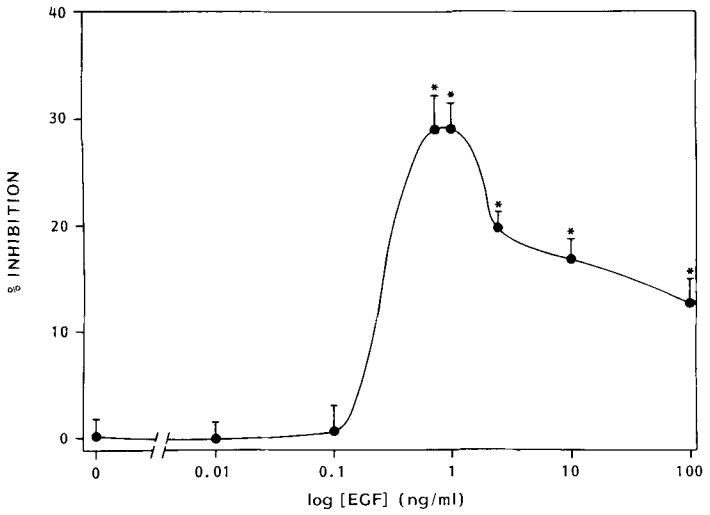

Fig. 1. Inhibition of basal $1,25(OH)_2D_3$ binding by increasing concentrations of EGF. Twenty-four hours after plating, the culture medium was changed to α-MEM with 2% charcoal-treated FCS and the cells were incubated for 24 h with or without EGF. Subsequently, the cells were incubated for an additional 4 h in serum-free α-MEM after which $1,25(OH)_2D_3$ binding was determined. $^*P < 0.001$ vs. control $1,25(OH)_2D_3$ binding.

h in α-MEM supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS. After 24 h, the medium was replaced by α-MEM with 2% charcoal-treated FCS and the cells were cultured for another 24 h period during which the cells reached confluence. The cells were treated with EGF during this second 24 h culture period. Except for the time-course studies, the cells were preincubated for the entire 24 h with EGF. After this preincubation period with EGF, the medium was changed to serum-free α-MEM and the cells were incubated for an additional 4 h ($1,25(OH)_2D_3$ receptor study) or 3 min (cAMP production study) with or without PTH, forskolin, PGE_2, Bt_2cAMP, or IBMX. Both 4 h and 3 min resulted in a maximal effect of the drugs tested on $1,25(OH)_2D_3$ binding and cAMP production, respectively. In another experimental set-up the cells were incubated at confluence for various periods with EGF after which $1,25(OH)_2D_3$ binding was assayed. All cell culture and incubation procedures were carried out at 37°C under 5% CO_2 and 95% air.

Preparation of Cell Extracts and $1,25(OH)_2D_3$ Binding Assay

For single point assays, conditions were used which were previously shown to provide valid estimates of total receptor content in cytosolic extracts [27]. The cell pellet was extracted on ice in a hypertonic buffer consisting of 300 mM KCl, 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 5 mM dithiotreitol, 10 mM sodium molybdate, and 0.1% Triton X-100. High-speed supernatants were obtained and 200 μl aliquots were incubated at 0°C overnight with 0.5 nM [^{3}H]$1,25(OH)_2D_3$ in the absence or presence of a 200-fold molar excess of unlabeled hormone. Receptor bound $1,25(OH)_2D_3$ was separated from unbound sterol by charcoal adsorption [28]. The protein concentration was measured according to the method of Bradford [29]. Changes in DNA content were assessed by the fluorimetrical method of Johnson-Wint and Hollis [30].

Measurement of cAMP

The incubation with the agents to be tested was stopped by removing the incubation medium followed by extraction of cAMP from the cells with 1 ml 90% isopropanol. cAMP was measured by the protein binding assay of Brown et al. [31].

Data Analysis

Data presented are means ± SD of triplicate determinations of at least two different experiments, i.e., at least six replicates. Multiple comparisons were performed using the one-way analysis of variance. Other statistical analyses were done by Student's *t* test.

Results

The effect of EGF on basal $1,25(OH)_2D_3$ binding is shown in Figure 1. Maximum inhibition of $1,25(OH)_2D_3$ binding (approx. 30%) was found at 1 ng/ml EGF. Preincubation experiments revealed that 3 h preincubation with 1 ng/ml EGF is sufficient to induce down-regulation. Maximal down-regulation was reached after 4 h preincubation and remained constant up to 24 h (data not shown). As stated in the Materials and Methods, it is important to notice that the preincubation with EGF was followed by an additional incubation for 4 h in serum-free medium in the absence or presence of PTH. When incubation for 4 h with EGF (0.01–10 ng/ml) was not followed by an additional incubation period, no decrease basal $1,25(OH)_2D_3$ binding was observed. In this experimental set-up, however, longer incubation periods with EGF, tested up to 48 h, did result in a decrease of $1,25(OH)_2D_3$ binding (data not shown). These data suggest that EGF can initiate cellular activity and then be removed without affecting the eventual cellular response.

Scatchard analysis showed that EGF induced a decrease in saturable $1,25(OH)_2D_3$ binding sites without a significant change in the apparent dissociation constant (15–20 pM) of $1,25(OH)_2D_3$ binding (Fig. 2A and B). Preincubation for 24 h with EGF did not significantly effect either DNA and protein content (data not shown).

Recently, we have shown that PTH dose-dependently stimulates $1,25(OH)_2D_3$ binding [11]. As depicted in Figure 3, 24 h preincubation with EGF resulted in a dose-dependent reduction of the stimulation of $1,25(OH)_2D_3$ binding by PTH. Significant inhibition was already observed at 0.01 ng/ml while a maximum inhibition of 30% was reached at 1 ng/ml EGF. The IC_{50} was about 0.1 ng/ml. Figure 4 shows the time-dependence of preincubation with 1 ng/ml EGF. The minimal preincubation time before significant inhibition of PTH-stimulated $1,25(OH)_2D_3$ binding could be observed was 2 h. Preincubation for 2 h to at least 24 h resulted in maximal inhibition. When 1 ng/ml EGF was added simultaneously with 10 nM PTH at the start of the 4 h incubation period, EGF was without effect (data not shown).

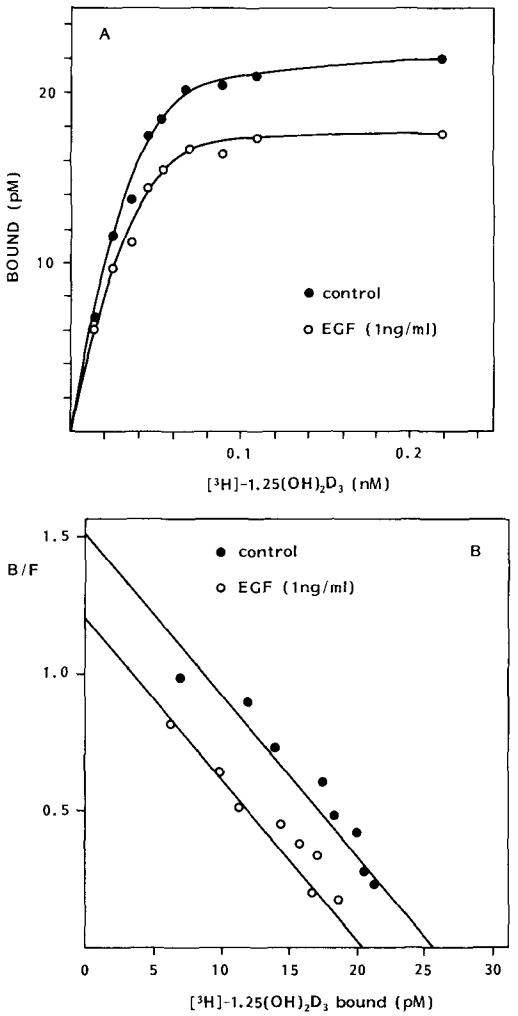

Fig. 2. (**A**) Saturation and (**B**) Scatchard analyses of $1,25(OH)_2D_3$ binding after treatment with vehicle or 1 ng/ml EGF. Vehicle and EGF were added 24 h after plating and 24 h before $1,25(OH)_2D_3$ binding was determined. Receptor content in cell extracts was determined as described in the Materials and Methods. Data in A were used for Scatchard analysis in B.

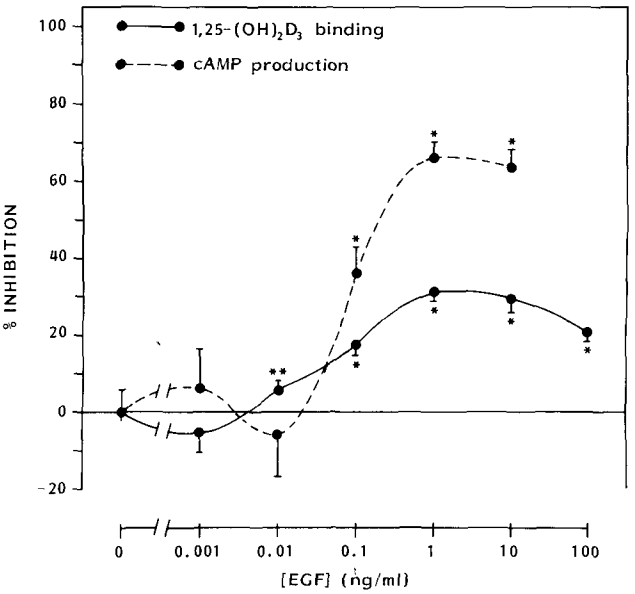

Fig. 3. Inhibition of PTH-stimulated $1,25(OH)_2D_3$ binding and cAMP production by increasing concentrations of EGF. Twenty-four hours after plating, the culture medium was changed to α-MEM with 2% charcoal-treated FCS and the cells were incubated for 24 h with or without EGF. Subsequently, the cells were incubated for an additional 4 h or 3 min in serum-free α-MEM with or without 10 nM PTH after which $1,25(OH)_2D_3$ binding and cAMP content, respectively, were determined as described in Materials and Methods. $^{**}P < 0.05$, $^{*}P < 0.001$ vs. effect of 10 nM PTH on $1,25(OH)_2D_3$ binding and cAMP content after preincubation with control medium.

The stimulation of $1,25(OH)_2D_3$ binding by PTH is preceded by a stimulation of cAMP production [11]. As presented in Figure 3, inhibition of PTH-stimulated $1,25(OH)_2D_3$ binding by EGF is accompanied by an inhibition of PTH-stimulated cAMP production. Both maximal inhibitory EGF concentration (1 ng/ml) and IC_{50} (0.1 ng/ml) are similar for $1,25(OH)_2D_3$ binding and cAMP production. However, three differences between inhibition of $1,25(OH)_2D_3$ binding cAMP production were observed: (1) significant inhibition of $1,25(OH)_2D_3$ binding was already observed at 0.01 ng/ml EGF in contrast to 0.1 ng/ml for inhibition of cAMP production; (2) maximal inhibition of cAMP production was 65% instead of 30% for the $1,25(OH)_2D_3$ binding; and (3) time-course experiments revealed that significant inhibition of PTH-stimulated $1,25(OH)_2D_3$ receptor up-regulation was observed after 2 h preincubation whereas PTH-stimulated cAMP production was first significantly reduced after 6 h preincubation with 1 ng/ml EGF (Figs. 4 and 5). The present study did not show whether the effect on PTH-stimulated cAMP production after 24 h preincubation represents the maximal inhibition. Routinely the cAMP content was measured after 3 min incubation with PTH, whereas the $1,25(OH)_2D_3$ binding was assayed after 4 h incubation with PTH. We therefore measured the cAMP content after 4 h treatment with PTH. These experiments showed that after 4 h incubation with 10 nM PTH, the cAMP content is still lower in EGF-treated cells (data not shown).

In order to examine whether EGF affects the ED_{50} of the PTH effect on the number of $1,25(OH)_2D_3$ binding and cAMP production, we performed a PTH dose-response study. This study revealed that preincubation with EGF did not result in a marked change of the ED_{50} for both PTH responses but did reduce the maxmal response (Figs. 6A and B). Moreover, as can be seen in Figures 1, 3, and 6A, 1 ng/ml EGF but not 0.01 ng/ml decreased basal $1,25(OH)_2D_3$ binding whereas PTH-stimulated $1,25(OH)_2D_3$ binding is already reduced by 0.01 ng/ml EGF. Furthermore, comparison of Figures 6A and B shows once again that 0.01 ng/ml EGF did not inhibit the PTH-stimulated cAMP production.

In contrast to the inhibition by EGF of PTH-stimulated $1,25(OH)_2D_3$ binding, the inhibition of basal $1,25(OH)_2D_3$ binding was not paralleled by a change in cAMP content. After 24 h preincubation, none of the EGF concentrations tested (0.001–10 ng/ml) affected basal cAMP concentration (data not shown). Also, as shown in Figure 5, different preincubation periods with 1 ng/ml EGF did not result in a change of basal cAMP content.

Next we studied whether the inhibitory effect of EGF was specific for PTH. The effects of 24 h preincubation with 1 ng/ml EGF on 10 μM PGE_2- and 10 μM forskolin-stimulated cAMP production and $1,25(OH)_2D_3$ binding are

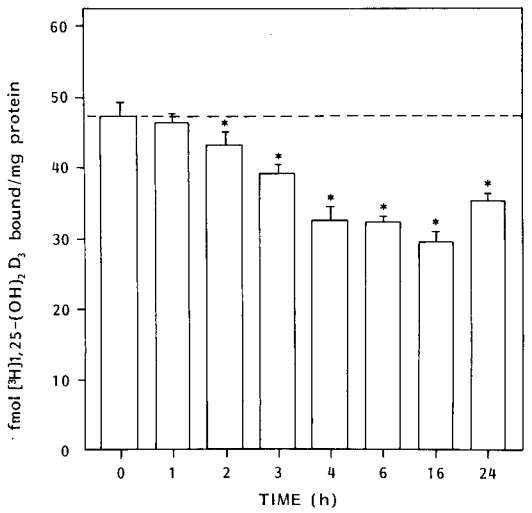

Fig. 4. Time-course of effects of preincubation with EGF on PTH-stimulated $1,25(OH)_2D_3$ binding. Twenty-four hours after plating, the culture medium was changed to α-MEM with 2% charcoal-treated FCS and the cells were incubated for varying periods of time with 1 ng/ml EGF. The incubation with EGF was followed by an additional 4 h incubation with or without 10 nM PTH in serum-free α-MEM. $1,25(OH)_2D_3$ binding was assessed as described in Materials and Methods. $^*P < 0.001$ vs. $1,25(OH)_2D_3$ binding after preincubation for the same period without EGF.

shown in Figure 7A and B, respectively. Both PGE_2- and forskolin-stimulated cAMP production are dose-dependently inhibited by EGF with a similar maximally effective concentration (1 ng/ml and IC_{50} (0.1 ng/ml) as for the inhibition of PTH-stimulated cAMP production. Also, PGE_2-stimulated $1,25(OH)_2D_3$ binding is affected with the same maximally effective concentration and IC_{50} as PTH-stimulated $1,25(OH)_2D_3$ binding (Fig. 7A). Forskolin (10 μM)-stimulated $1,25(OH)_2D_3$ binding is also inhibited by EGF but the degree of inhibition by 1 and 10 ng/ml EGF is less than that of PTH and PGE_2 (Fig. 7B).

Stimulation of $1,25(OH)_2D_3$ binding by direct activation of protein kinase A with 1.5 mM Bt_2cAMP was not reduced by 24 h preincubation with 1 ng/ml EGF (Fig. 8). Addition of the cAMP phosphodiesterase inhibitor IBMX resulted in a modest increase of $1,25(OH)_2D_3$ binding and cAMP content. Both responses were not inhibited by preincubation with EGF (data not shown).

$1,25(OH)_2D_3$ causes a homologous up-regulation of its binding without a change in cAMP concentration [27]. As depicted in Table 1, this homologous up-regulation is not affected by preincubation with various concentrations of EGF.

Discussion

The present study shows a direct effect of EGF on $1,25(OH)_2D_3$ receptor level in the osteoblast-like cell line UMR 106. In other studies with various osteoblast-like cells, EGF has been shown to decrease alkaline phosphatase activity, hydroxyproline content, and collagen synthesis [16, 19]. Among the osteosarcoma cell lines, there is a close relation between, on the one hand, the presence of receptors for and biological responses to $1,25(OH)_2D_3$ and on the other hand, osteoblastic properties such as elevated alkaline phosphatase activity and bone formation in subcutaneous tumors [32]. Therefore, it is tempting to suggest that EGF shifts osteoblasts to cells with a less differentiated phenotype. EGF has a proliferative effect on osteoblasts, as judged by an increase of DNA synthesis, [^{3}H]-thymidine incorporation, and cell number [16, 17, 19]. In the present study, no effect on DNA synthesis and protein content was observed after incubation with EGF. This discrepancy could be due to the relative short incubation period (24 h) in our studies.

In a human breast epithelial cell, HBL 100, EGF decreases glucocorticoid binding [24], and in Leydig tumor cells EGF decreases gonadotropin receptor number [25]. Despite several differences, a clear resemblance between the effects of EGF on the receptor binding of $1,25(OH)_2D_3$ and these two hormones is that EGF does not change receptor affinity but only seems to cause a change in the number of binding sites (Figs. 2A and B).

For the decrease of glucocorticoid binding, a causal role for EGF-dependent protein tyrosine kinase was proposed [24]. Indeed, recent evidence supports tyrosine phosphorylation of human glucocorticoid receptor by EGF [33]. In view of the fact that in our experiments inhibition of $1,25(OH)_2D_3$ binding was only observed after incubation periods exceeding 4 h, it is not likely that a direct phosphorylation of the $1,25(OH)_2D_3$ receptor by EGF-receptor tyrosine kinase is involved.

EGF has been shown to modulate cellular responses to several hormones in various cell types [21–25]. Recently, it has been reported that pretreatment of UMR 106 cells with EGF inhibits the cellular cAMP response to PTH [20]. In these cells the PTH-stimulated $1,25(OH)_2D_3$ binding is preceded by an increase in cAMP production [11]. The present study shows that inhibition of PTH-stimulated cAMP production is accompanied by an attenuation of the PTH-stimulated increase of $1,25(OH)_2D_3$ binding sites (Fig. 3). This observation is in contrast to the effect of EGF on basal $1,25(OH)_2D_3$ receptor level which is not accompanied by a change in cellular cAMP content (Figs. 1 and 5). A remarkable difference between the inhibition of PTH-stimulated cAMP response and $1,25(OH)_2D_3$ receptor up-regulation concerns the magnitude of the maximal inhibition, 65 and 30%, respectively. However, if one plots cAMP content against the number of $1,25(OH)_2D_3$ binding sites, a 65% inhibition of the PTH-stimulated cAMP production still leaves an absolute cAMP content sufficient to maintain a stimulation of $1,25(OH)_2D_3$ binding by PTH of approximately 70% of the maximal stimulation of $1,25(OH)_2D_3$ binding by PTH.

For human choriogonadotropin-stimulated steroidogenesis in cultured Leydig tumor cells, two opposing effects, dependent on the incubation period with EGF, have been described [25]. Based on the present data it is unlikely that, dependent on the incubation period, EGF has opposing effects on PTH responses in UMR 106. In our cells, EGF reduced both PTH-stimulated $1,25(OH)_2D_3$ binding and cAMP generation without an apparent change in sensitivity to PTH (Figs. 6A and B). The effect of EGF on PTH-stimulated cAMP production is similar to the effect of transforming growth factor-α (TGFα), known to act via the EGF receptor, on PTH-stimulated cAMP production [20].

In an attempt to pinpoint the site of action of EGF, we tested its effect on $1,25(OH)_2D_3$ receptor up-regulation by direct stimulation of protein kinase A with Bt_2cAMP. As shown in Figure 8, Bt_2cAMP-stimulated $1,25(OH)_2D_3$ binding is not affected by EGF. The observed reduction is completely due to a reduction of basal $1,25(OH)_2D_3$ binding by EGF, as the absolute increase in $1,25(OH)_2D_3$ binding by

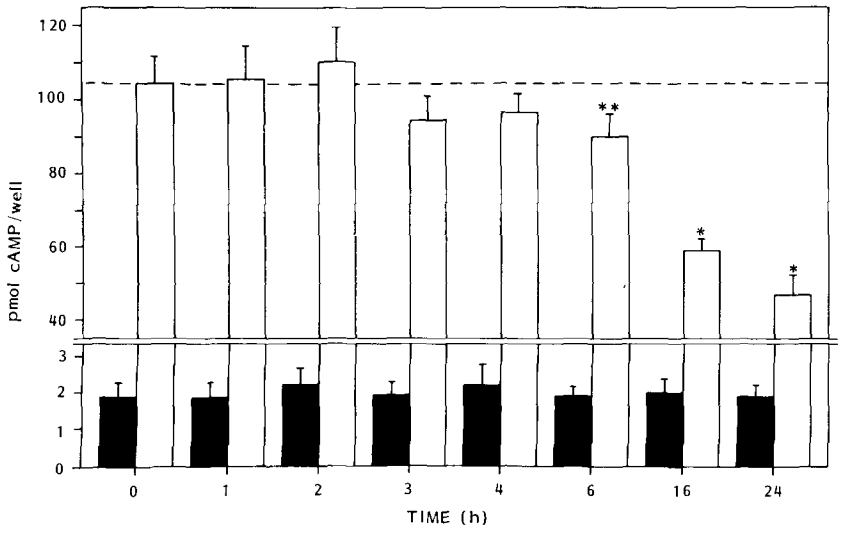

Fig. 5. Time-course of effects of preincubation with EGF on basal and PTH-stimulated cAMP production. Twenty-four hours after plating, the culture medium was changed to α-MEM with 2% charcoal-treated FCS and the cells were incubated for varying periods of time with 1 ng/ml EGF. The incubation with EGF was followed by an incubation for 3 min with or without 10 nM PTH after which the cAMP content was determined. $^{**}P < 0.05$, $^{*}P$ 0.001 vs. cAMP content after preincubation for the same period without EGF.

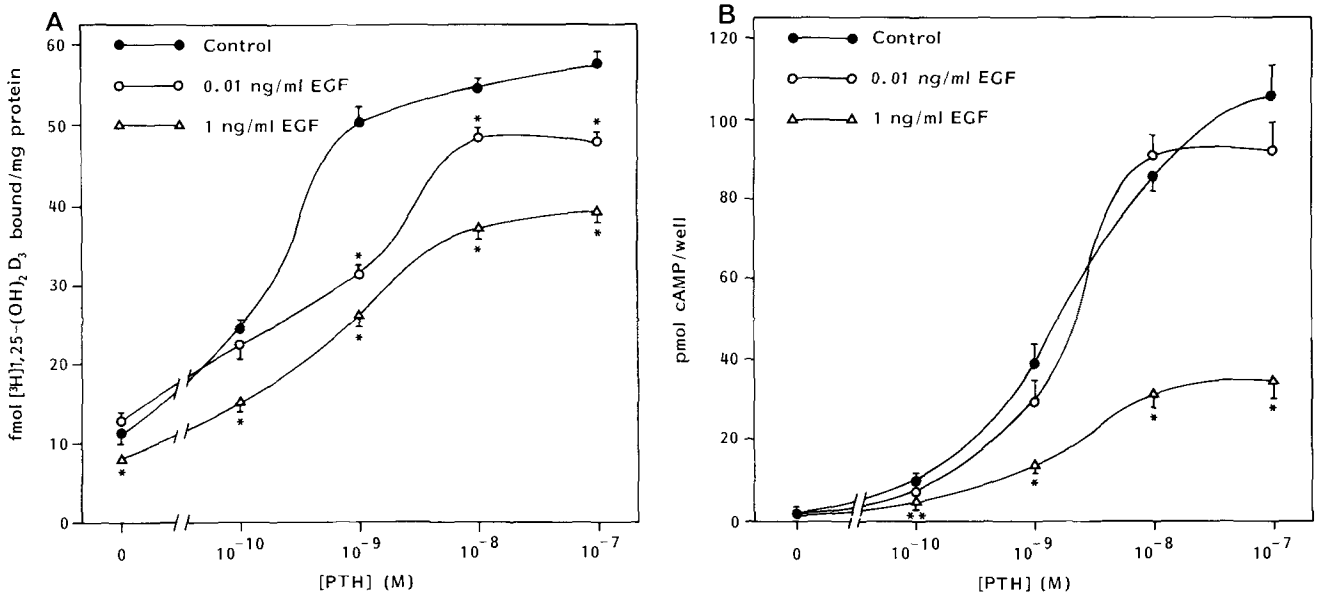

Fig. 6. Effect of EGF on PTH dose-response curves for **(A)** $1,25(OH)_2D_3$ binding and **(B)** cAMP production. Twenty-four hours after plating, culture medium was changed to α-MEM with 2% charcoal-treated FCS and the cells were incubated for 24 h with or without 0.01 or 1 ng/ml EGF. The incubation with EGF was followed by an additional incubation for 4 h or 3 min in serum-free α-MEM with varying concentrations of PTH after which $1,25(OH)_2D_3$ binding and cAMP content, respectively, were determined. $1,25(OH)_2D_3$ binding and cAMP content were assessed as described in Materials and Methods. $^{**}P < 0.01$, $^{*}P < 0.001$ vs. the effect of the same concentration of PTH after preincubation with control medium.

Bt_2cAMP remains constant over the whole range of EGF concentrations tested. Also the rise in $1,25(OH)_2D_3$ binding and cAMP content induced by the cAMP phosphodiesterase inhibitor IBMX were not affected by EGF. These data suggest that EGF exerts its effect at the level of cAMP production, i.e., the PTH receptor or the G-protein/adenylate cyclase complex.

To elucidate whether the EGF effect is located at the level of the PTH receptor we tested two other drugs known to stimulate $1,25(OH)_2D_3$ binding and to act via the cAMP-messenger system: PGE_2 and forskolin. Figure 7A and B show that inhibition of stimulated $1,25(OH)_2D_3$ binding and cAMP production by EGF is not specific for PTH. The forskolin-stimulated cAMP production and $1,25(OH)_2D_3$ binding are reduced in a similar way to the PTH and PGE_2 responses, although the maximal effect on forskolin-stimulated $1,25(OH)_2D_3$ binding is somewhat lower. These data indicate that the EGF effect is not primarily located at the PTH or PGE_2 receptor. However, conclusive data have to be provided by PTH and PGE_2 binding experiments which are part of forthcoming studies. Moreover, the facts that forskolin responses are also reduced by EGF and that forskolin is capable of stimulating adenylate cyclase without G-protein interaction [34] suggest a localization of the EGF effect at the catalytic unit of the adenylate cyclase. In both the UMR 106 cells [20] and the MA-10 Leydig tumor cells [25] it has

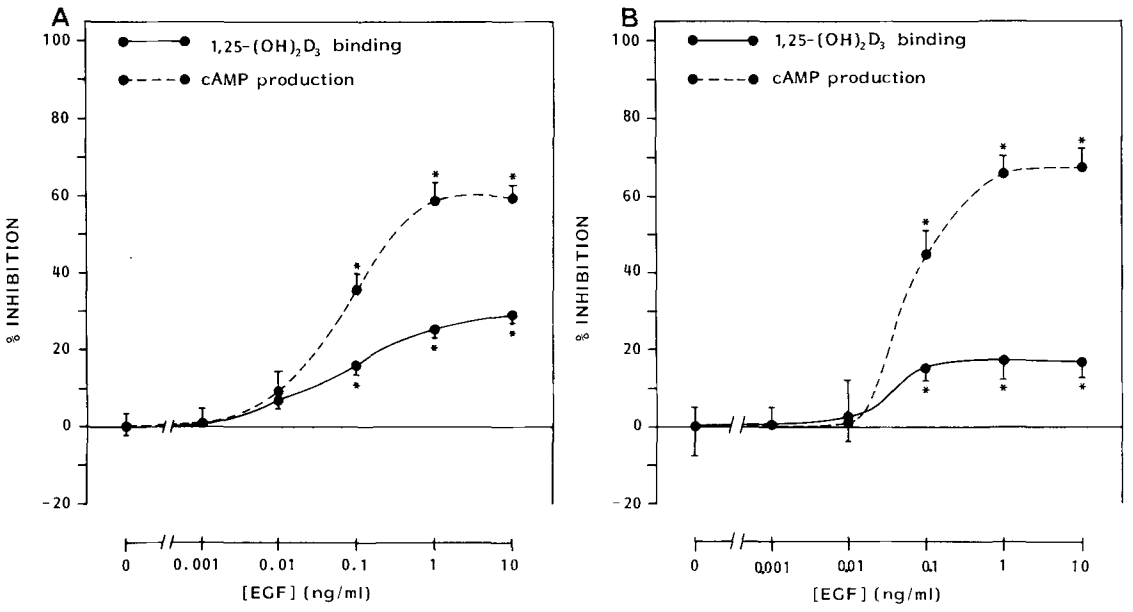

Fig. 7. Inhibition of (**A**) PGE_2- and (**B**) forskolin-stimulated $1,25(OH)_2D_3$ binding and cAMP production by EGF. Twenty-four hours after plating, the culture medium was changed to α-MEM with 2% charcoal-treated FCS, and the cells were incubated for 24 h with or without EGF. Subsequently, the cells were incubated for an additional 4 h or 3 min in serum-free α-MEM with or without 10 μM PGE_2 or 10 μM forskolin after which $1,25(OH)_2D_3$ binding and cAMP content, respectively, were determined as described in Materials and Methods. $^*P <$ 0.001 vs. effect of PGE_2 or forskolin on $1,25(OH)_2D_3$ binding and cAMP content after preincubation with control medium.

been shown that TGFα and EGF do not act on the G_i-protein. Whether G_s is a target for EGF action needs further studies. It has recently been demonstrated that TGFα inhibits the cholera toxin-stimulated cAMP production in UMR 106 cells [20]. In contrast to one of the results of this latter study by Gutierrez et al. [20] we did find that PGE_2 stimulated cAMP production is reduced by EGF. Whether this discrepancy represents differences in cell type or culture or incubation procedures is not clear.

The present study shows a close relation between the EGF-induced inhibition of heterologous up-regulation of the $1,25(OH)_2D_3$ binding sites and of cAMP production. This is supported by the finding that cAMP-independent homologous up-regulation of the $1,25(OH)_2D_3$ receptor is not affected by EGF (Table 1). However, the current data contain three observations that suggest an effect of EGF also on stimulated $1,25(OH)_2D_3$ binding independent of the cAMP messenger system. First, at 0.01 ng/ml, EGF inhibits $1,25(OH)_2D_3$ receptor up-regulation by PTH but not the stimulation of cAMP production (Figs. 3, 6A, and B). Second, comparison of Figures 4 and 5 reveals that preincubation with EGF for 2–4 h results in a decrease of PTH-stimulated $1,25(OH)_2D_3$ binding whereas the cAMP response to PTH is not affected. Thus, in both instances the generation of the second messenger signal is normal but the biological response is already reduced. Third, the maximal inhibition of cAMP production is similar for PTH, PGE_2, and forskolin whereas the degree of inhibition of $1,25(OH)_2D_3$ binding is twice as high for PTH and PGE_2 as for forskolin. All three agonists increase the intracellular ionized calcium concentration in osteoblastic cells [35–38]. Inositol 1,4,5-trisphosphate (IP_3) formation, and thereby calcium release from intracellular stores, is involved in the action of PTH and PGE_2 [35, 39, 40] whereas the effect of forskolin on the intracellular ionized calcium concentration is the result of an increased calcium influx [37, 38]. Indeed, in mouse osteoblast cultures, forskolin had no effect on basal levels of total

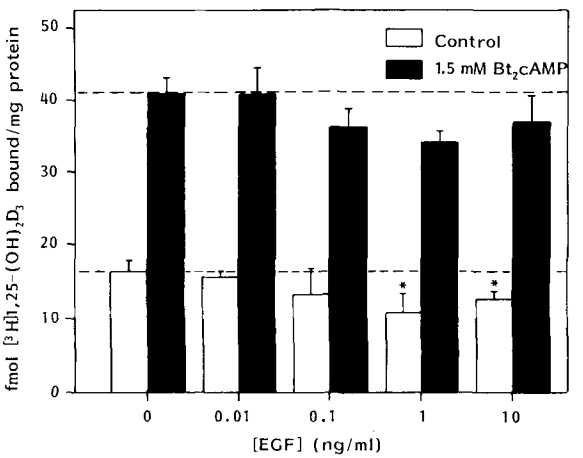

Fig. 8. Effect of EGF on Bt_2cAMP-stimulated $1,25(OH)_2D_3$ binding. Twenty-four hour plating, the culture medium was changed to α-MEM with 2% charcoal-treated FCS and the cells were incubated for 24 h with or without EGF. Subsequently, the cells were incubated for an additional 4 h in serum-free α-MEM with or without 1.5 mM Bt_2cAMP after which $1,25(OH)_2D_3$ binding was determined as described in Materials and Methods. $^*P < 0.001$ vs. the $1,25(OH)_2D_3$ binding after preincubation with control medium.

IP_3 accumulation [41]. Therefore, it would be interesting to study the effect of EGF on IP_3 formation and protein kinase C activation which could provide an explanation for the less potent effect of EGF on forskolin-stimulated $1,25(OH)_2D_3$ receptor up-regulation. In hepatocytes it has been shown that EGF stimulates formation of IP_3 and activates diacylglycerol- and Ca^{2+}-dependent protein phosphorylation [42, 43].

The present study shows that in the osteoblastic cell line

Table 1. Effect of EGF on homologous up-regulation of the $1,25(OH)_2D_3$ binding by 10 nM $1,25(OH)_2D_3$

EGF (ng/ml)	$1,25(OH)_2D_3$ receptor content (% of control)
0	100 ± 7.6
0.01	107 ± 16.9
0.1	100 ± 15.7
1	103 ± 12.5
10	96 ± 9.6

Twenty-four hours after plating, culture medium was changed to α-MEM with 2% charcoal-treated FCS and the cells were incubated for 24 h with or without EGF. Subsequently the cells were incubated for an additional 4 h in serum-free α-MEM with or without 10 nM $1,25(OH)_2D_3$, after which $1,25(OH)_2D_3$ binding was determined as described in the Materials and Methods

UMR 106 important interactions exist (1) between EGF and a steroid hormone, $1,25(OH)_2D_3$; and (2) between EGF and a polypeptide hormone, PTH. Interactions shown in the current study and in other studies [20, 44] indicate that at the level of the osteoblast a regulation mechanism exists between EGF, $1,25(OH)_2D_3$ and PTH. The effects of EGF described in the current study are exerted at EGF concentrations which are comparable with the physiological plasma concentrations of EGF. Therefore, the results presented here may have important physiological implications for the regulation of bone metabolism. However, whether the observed reduction of $1,25(OH)_2D_3$ binding by EGF also results in a reduced biological response to $1,25(OH)_2D_3$ is not yet clear and currently under investigation. Preliminary data point to a relation between changes in $1,25(OH)_2D_3$ binding and its biological responses. As various tumors produce TGFα [45, 46] the present data may also be significant for the understanding of the process of humoral hypercalcemia of malignancy.

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