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Estradiol induction of cAMP in breast cancer cells is mediated by foetal calf serum (FCS) and sex hormone-binding globulin (SHBG)

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Abstract

Plasma sex hormone-binding globulin (SHBG or SBP), the specific carrier for estradiol and androgens, after binding to its membrane receptor (SHBG-R), causes a significant increase of cAMP in the presence of estradiol, in both breast (MCF-7) and prostate (LNCaP) cancer cells maintained in serum-free medium. On the other hand, it has been proposed that estrogens, in addition to the well-known nuclear receptor pathway, exert their biological effect inducing cAMP, as a consequence of a direct membrane action, in breast cancer and uterine cells. The aim of the present study was to clarify this controversial issue by verifying if the cAMP increase in MCF-7 cells was a direct effect of estradiol, or if it was mediated by FCS proteins, such as bovine sex hormone-binding globulin; and to reevaluate the effect of human SHBG on cAMP induction in the presence of FCS. MCF-7 cells were maintained in DCC-FCS (treated with DCC to remove steroids), in SHBG-FREE/DCC-FCS (treated with DCC and with a specific affinity chromatography to remove bovine sex hormone-binding globulin), or in serum-free medium (SFM). It was observed that estradiol determined a significant time-dependent increase of cAMP only in MCF-7 cells maintained in 10% DCC-FCS. When cells were maintained in 10% SHBG-FREE/DCC-FCS, estradiol had no detectable effect. However, its ability to increase cAMP was observed again after the addition of human SHBG, in doses ranging from 5 to 50 nM. Moreover, in the presence of 10% SHBG-FREE/DCC-FCS, SHBG, even in the absence of estradiol, caused a significant increase of cAMP. In conclusion, the data reported in the present study suggest that the ability of estradiol to induce cAMP in MCF-7 cells is not due to a direct membrane effect of the hormone, but rather it is mediated by FCS. SHBG is one of the serum factors mediating estradiol action. Lastly, it was proven that SHBG triggers the cAMP pathway in MCF-7 cells in a *physiologic* culture condition and at *physiologic* concentrations.

1. Introduction

Human plasma sex hormone-binding globulin (SHBG or SBP), the specific carrier for androgens and estradiol, also binds to a specific receptor (SHBG-R) on membranes of estrogen- and androgen-dependent tissues, such as breast [1] and prostate [2] cancer. The interaction between SHBG and SHBG-R in MCF-7 cells is involved in the control of estradiol action. In fact, SHBG inhibits the estradiol-induced proliferation of MCF-7 cells [3,4] using cAMP as second messenger and, consequently, the activation of PKA. The ability of SHBG to induce cAMP was investigated in breast [5] and prostate [6–8] cancer cells. In both cell types, SHBG increased the intracellular cAMP level only in the presence of sex steroids (e.g. estradiol), while either estradiol or SHBG alone had no detectable effect on cAMP. On the contrary, other authors [9] described how estradiol alone (without added human SHBG) was able to induce cAMP both in MCF-7 cells and in primary cultures of uterine cells. The discrepancy in

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the data reported by different laboratories could be due to the different experimental procedures. In fact, all the studies related to SHBG and SHBG-R were performed in serum-free medium, in order to avoid any interference from the bovine sex hormone-binding globulin (which is present in relatively high concentrations in serum normally used in cell cultures). On the other hand, the study by Aronica et al. was performed in the presence of serum treated with DCC (dextran-coated charcoal), which removes steroids, but does not remove proteins such as the bovine sex hormone-binding globulin.

Since the understanding of the actual mechanism causing estradiol to induce cAMP in breast cancer cells (direct membrane action vs. SHBG and SHBG-R mediated action) is of critical importance, we decided to go back to our previous data on SHBG and cAMP and to reevaluate the protein's activity. In the present study, we report the ability of the SHBG/ SHBG-R system to increase cAMP in MCF-7 cells, maintained in modified FCS, in which either steroids and bovine sex hormone-binding globulin were removed, using specific methods. Lastly, the ultimate purpose of the study was to understand the previous discrepancies and to observe the SHBG effect in a more *physiologic* environment for cultured breast cancer cells.

2. Materials and methods

2.1. Materials

MCF-7 cells (estrogen-dependent breast cancer cells), [10], were a gift of C. Dati (Dept. Biologia Animale, University of Torino). Foetal Calf Serum (FCS) was purchased from Hyclone (Logan UT, USA), L-glutamine and trypsin-EDTA from Gibco Brl (Gaithesburg MD, USA), cell culture disposable materials from Corning (New York NY, USA). RPMI 1640 with and without phenol red, insulin-transferrin-Na Selenite (ITS), bovine serum albumin solution (BSA), radioinert estradiol and dihydrotestosterone and testosterone-agarose were purchased from Sigma Chemical (St. Louis MO, USA). The AMPREP SAX columns, the cAMP [^{125}I] assay system (dual range) and dihydrotestosterone labeled with ^{3}H (^{3}H-DHT) were purchased from Amersham Pharmacia Biotech Italia (Cologno Monzese, MI, Italy).

All other reagents were analytical grade.

2.2. Preparation of DCC-FCS and SHBG-FREE/DCC-FCS

FCS from the same batch was first tested for bovine sex hormone-binding globulin content with a specific binding assay [11] and for estradiol with a commercial radioimmunoassay. Steroids were then removed with a slurry of dextran (0.5%) charcoal (0.05%), (DCC), [9]. A portion of the DCC-FCS was additionally treated in order to remove bovine sex hormone-binding globulin. Briefly, DCC-FCS was passed through a testosterone-agarose column used in our laboratory for SHBG purification. Serum eluting from the column was additionally treated with DCC and concentrated back to its initial volume. Estradiol and sex hormone-binding globulin concentrations were tested at the end of the procedure. FCS, DCC-FCS and SHBG FREE/DCC-FCS were aliquoted and maintained at −20°C until used.

2.3. Cell culture

MCF-7 cells (estrogen-dependent breast cancer cultured cells) were routinary maintained in RPMI 1640 and 10% heat inactivated FCS, 100 IU/ml Penicillin, 100 μg/ml Streptomycin, at 37°C, 5% CO_2, 95% humidity. Seven to 10 days before experiments, the cells were seeded in RPMI 1640 without phenol red supplemented with 10% whole FCS; 48 h later, the medium was changed with RPMI 1640 without phenol red supplemented with 10% of DCC-FCS; 48 h later, the medium was switched to a medium supplemented with 10% of SHBG-FREE/DCC-FCS. The cells were then seeded in appropriate vessels for the experiments in RPMI 1640 without phenol red, supplemented with 10% SHBG-FREE/DCC-FCS, and after 48 h were switched to the appropriate medium (either RPMI 1640 without phenol red, supplemented with 5 or 10% DCC-FCS or SHBG-FREE/DCC-FCS, or serum-free medium, SFM, prepared, as previously described [4], with RPMI 1640 without phenol red, 1 μg/ml insulin, 1 μg/ml transferrin, 1 ng/ml Na selenite, 0.5% BSA).

2.4. cAMP evaluation

MCF-7 cells were seeded in 6-well multiplates either in RPMI 1640 without phenol red supplemented with 5 or 10% of DCC-FCS or SHBG-FREE/DCC-FCS or in SFM. After 48 h from seeding, treatments were initiated as follows:

1. Effect of DCC-FCS and estradiol. To evaluate the time-course of the estradiol effect on cAMP generation, MCF-7 cells in 5 or 10% DCC-FCS, or in SFM, were treated with 1 nM estradiol (control cells were maintained in the medium without estradiol throughout the experiment) for different times ranging from 15 to 360 min at 37°C with gentle shaking. In another set of experiments, cells in 5 or 10% DCC-FCS, or in SFM, were treated for 15 or 180 min with increasing estradiol concentrations ranging from 0 to 100 nM (at 37°C with gentle

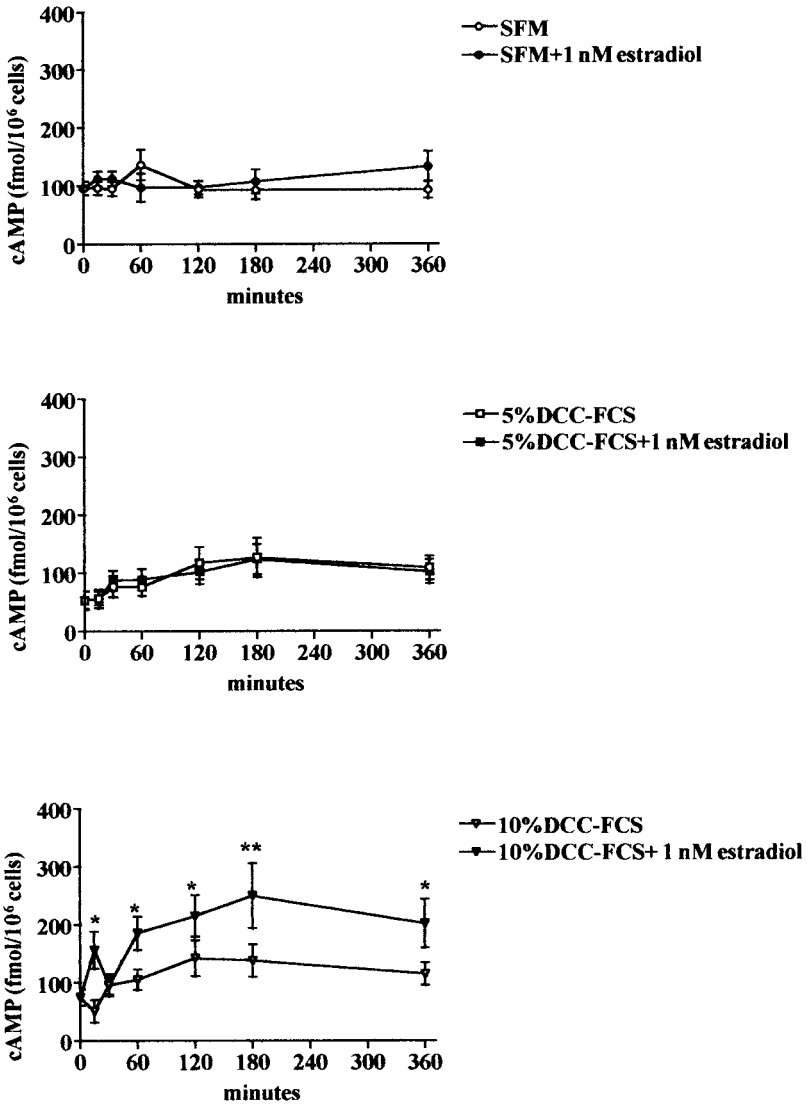

Fig. 1. cAMP induction in MCF-7 cells: effect of estradiol and DCC-FCS. cAMP was measured on extracts obtained from MCF-7 cells treated with 1 nM estradiol for different times (0–360 min) in SFM (serum-free medium, control), 5% DCC-FCS (FCS treated with charcoal to remove steroids) and 10% DCC-FCS. Data are expressed as mean ± S.E.M. obtained from three experiments. For each condition, data obtained in the absence and in the presence of estradiol were compared as described. Only significant statistical differences are reported as $^{*}P < 0.05$ and $^{**}P < 0.01$.

shaking), in order to test the most effective estradiol concentration on cAMP generation.

2. Effect of SHBG-FREE/DCC-FCS, estradiol and human SHBG. Cells in 5 or 10% SHBG-FREE/DCC-FCS, or SFM, were treated for 15 min (at 37°C with gentle shaking) with increasing estradiol concentrations ranging from 0 to 100 nM, preceded by a 40-min treatment with 50 nM human SHBG (kindly provided by G.L. Hammond, London Cancer Labs., London Ont., Canada). The control cells were treated with the same estradiol doses without SHBG pretreatment. In addition, in order to characterize the mechanism of action of SHBG in SHBG-FREE/DCC-FCS, cells in 10% SHBG-FREE/DCC-FCS were treated for 15 min (at 37°C with gentle shaking) with increasing estradiol concentrations ranging from 0 to 100 nM, preceded by a 40 min pretreatment with human SHBG in concentrations ranging from 0 to 50 nM.

Following the different treatments, cells were put in an ice-bath, mechanically detached from the vessel and resuspended in 1.0 ml of sterile PBS. The cell suspensions were passed through AMPREP SAX columns and the cAMP was extracted following the manufac-

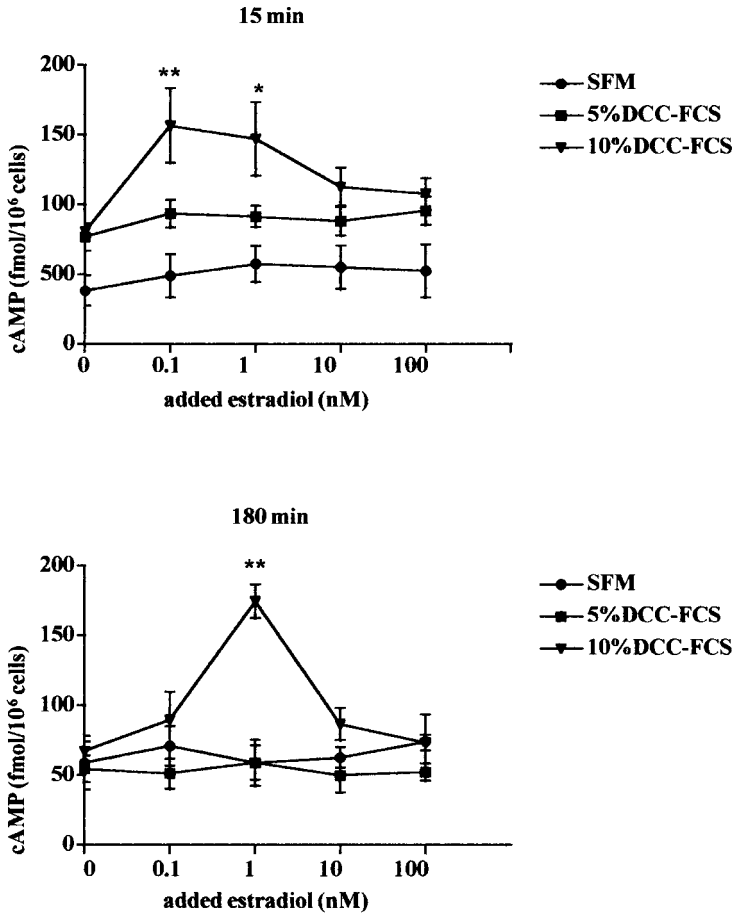

Fig. 2. cAMP induction in MCF-7 cells: effect of estradiol and DCC-FCS. cAMP was measured on extracts obtained from MCF-7 cells treated with increasing concentrations of estradiol (ranging from 0.1 to 100 nM) for 15 and 180 min in SFM (serum-free medium, control), 5% DCC-FCS (FCS treated with charcoal to remove steroids) and 10% DCC-FCS. Data are expressed as mean ± S.E.M. obtained from three experiments. Only significant statistical differences are reported as $^{*}P < 0.05$ and $^{**}P < 0.01$.

turer's instructions. The cAMP concentration of each extract was evaluated with the Amersham cAMP [^{125}I] assay system.

2.5. Statistical analysis

All analyses were performed using the PC program GRAPHPAD INSTAT (Graphpad Software). Data are expressed as mean ± S.E.M., obtained from a minimum of three experiments. Differences between means in compared groups were evaluated using ANOVA followed by the Bonferroni test, and statistical significance was attained for $p < 0.05$.

3. Results

3.1. Preparation of SHBG-FREE/DCC-FCS

Treatment of FCS with DCC, followed by chromatography on testosterone-agarose, determined an almost complete disappearance of estradiol and bovine sex hormone-binding globulin (in one typical experiment, estradiol, from 75 pg/ml to 2.2 pg/ml, 97% was removed; bovine sex hormone-binding globulin from 1266 c.p.m./tube specifically bound DHT to 64 c.p.m./tube specifically bound DHT, 95% was removed).

3.2. cAMP evaluation

The effect of estradiol alone on cAMP generation was evaluated first. As shown in Fig. 1, 1 nM estradiol was not able to increase cAMP content of MCF-7 cells maintained in SFM and 5% DCC-FCS, whereas it increased cAMP significantly in cells treated with 10% DCC-FCS. The effect was already evident after 15 min and increased over time reaching its maximum level after 3 h. This result was further confirmed by a successive experiment in which the effect of increasing estradiol doses was investigated for both times (Fig. 2). The estradiol effect was significant at the lowest doses we examined (0.1–1 nM). Thus, we concluded that estradiol was able to generate cAMP in MCF-7 cells in the presence of unknown serum factors that

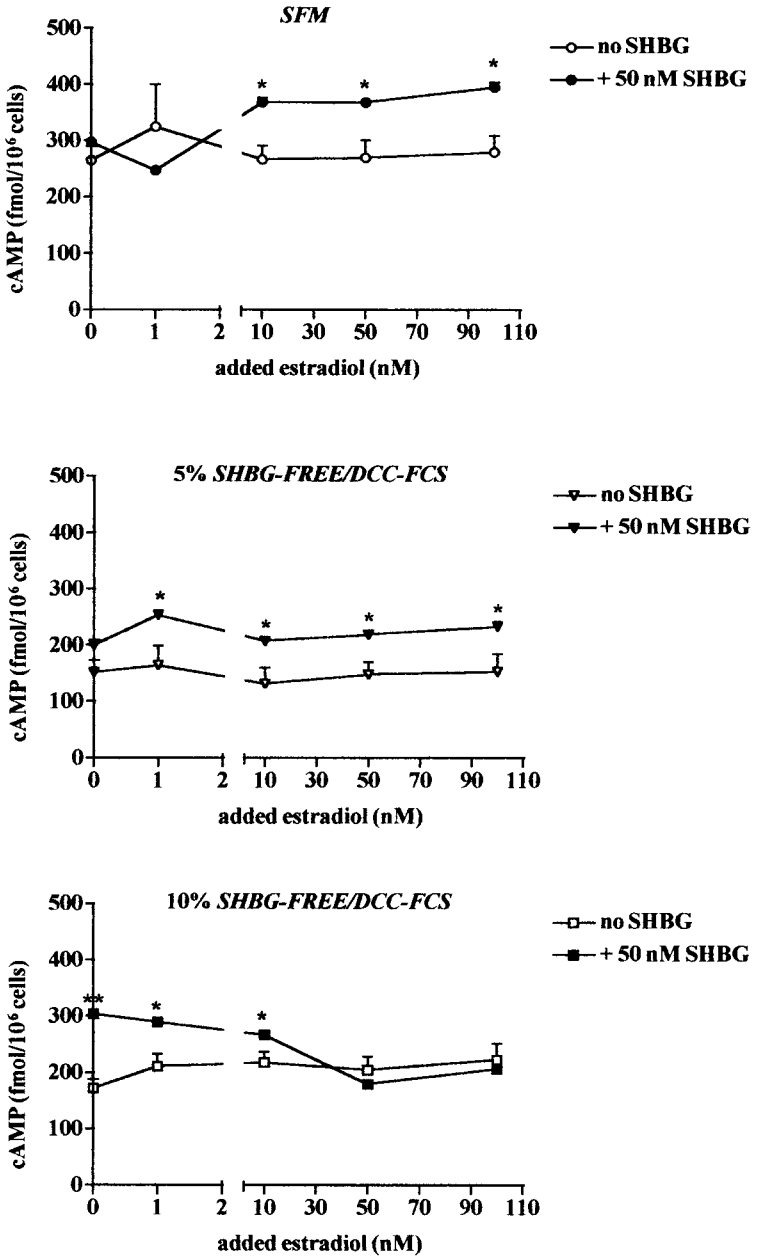

Fig. 3. cAMP induction in MCF-7 cells: effect of SHBG-FREE/DCC-FCS and SHBG. cAMP was evaluated on extracts obtained from MCF-7 cells treated with increasing estradiol concentrations (ranging from 1 to 100 nM) in the absence and in the presence of 50 nM human SHBG for 15 min in SFM (serum-free medium), 5% SHBG-FREE/DCC-FCS (DCC-FCS in which bovine sex hormone-binding globulin was removed) and 10% SHBG-FREE/DCC-FCS. Data are expressed as mean ± S.E.M. obtained from three experiments. Only significant statistical differences are reported as $^{*}P < 0.05$ and $^{**}P < 0.01$.

were likely to mediate estradiol action on adenylate cyclase. Since the effect observed after 15 min confirmed previous reports [5–7], and it was more consistent with the biological meaning of cAMP induction, the following experiments were conducted using this time period.

In order to understand whether human SHBG could be one of those serum factors able to mediate estradiol action on cAMP, we studied the effect of the hormone in cells treated with SHBG-FREE/DCC-FCS (in which bovine sex hormone-binding globulin had been removed, as previously described). As shown in Fig. 3, estradiol alone was not able to induce cAMP, regardless of the concentration of serum. Pretreatment with 50 nM human SHBG determined a significant increase of cAMP in all the culture conditions. The effect was more prominent in MCF-7 cells maintained in SFM or in 5% SHBG-FREE/DCC-FCS. In cells treated with 10% SHBG-FREE/DCC-FCS, cAMP increased only at the two lowest estradiol concentrations, in the pre-

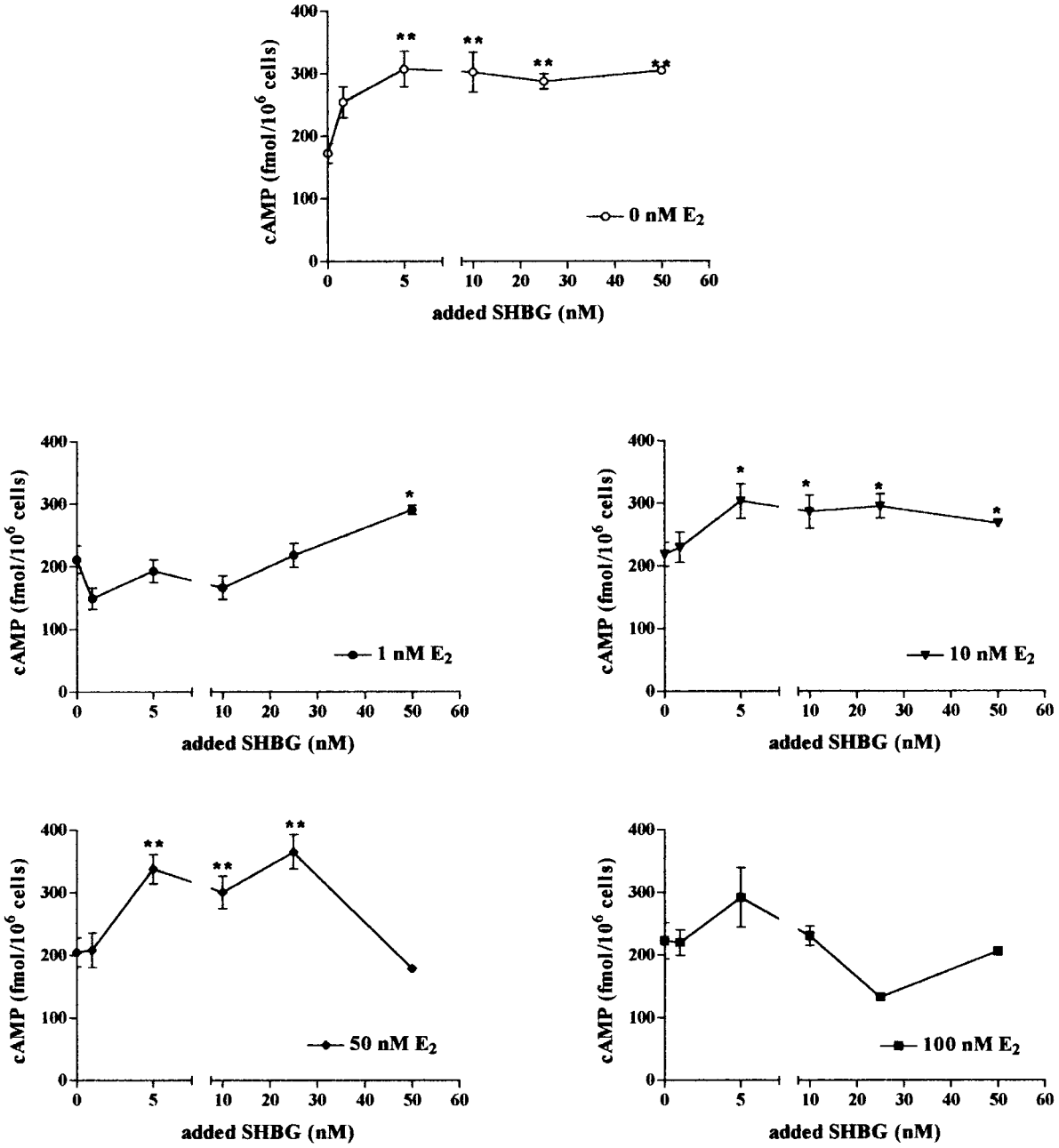

Fig. 4. cAMP induction in MCF-7 cells: effect of SHBG, estradiol and 10% SHBG-FREE/DCC-FCS. cAMP was evaluated on extracts obtained from MCF-7 cells, maintained in 10% SHBG-FREE/DCC-FCS, and treated with increasing human SHBG concentrations (ranging from 0 to 50 nM) in the absence and in the presence of increasing estradiol concentrations (ranging from 1 to 100 nM). Data are expressed as mean ± S.E.M. obtained from three experiments. Only significant statistical differences are reported as $^{*}P < 0.05$ and $^{**}P < 0.01$.

sence of human SHBG. In this last condition, the pretreatment with 50 nM human SHBG was effective in significantly increasing cAMP, even in the absence of estradiol.

The ability of SHBG to increase cAMP in cells maintained in 10% SHBG-FREE/DCC-FCS was further examined, as reported in Fig. 4. Human SHBG, even in the absence of estradiol, was confirmed to cause cAMP accumulation in MCF-7 cells. The lowest effective concentration of SHBG was 5 nM. Higher concentrations were effective as well, but no further increase was observed. In the presence of 1 nM estradiol, only 50 nM SHBG determined a significant increase of the second messenger. At 10 nM estradiol, all the SHBG concentrations (except 1 nM) were effective, while at 50 nM estradiol, only SHBG doses ranging from 5 to 25 nM were able to increase cAMP. The highest estradiol concentration we tested (100 nM) was shown not to cause cAMP accumulation in MCF-7 cells whatever the SHBG concentration was.

4. Discussion

The observations in this study provide clear evidence that the ability of estradiol to generate cAMP in MCF-7 breast cancer cells depends on serum factors, among which SHBG plays a critical role.

In both MCF-7 [4,5] and prostate cells [6–8], the induction of cAMP by SHBG and steroids had previously been investigated, in order to understand the signal transduction pathway triggered by the activation of the SHBG membrane receptor (SHBG-R). The binding of estradiol or androgens to the preformed SHBG/SHBG-R complex determined a significant accumulation of the second messenger, while steroid hormones alone had no such effect. On the other hand, Aronica et al. [9], on the basis of the observation that estradiol alone increased cAMP in MCF-7 and uterine cells in DCC-FCS supplemented medium, suggested a possible membrane action for estradiol. One possible explanation for this discrepancy was related to the use of FCS during experiments. In fact, the laboratories dealing with SHBG had always been worried about the interferences exerted by bovine sex hormone-binding globulin, which is present at relatively high concentrations in FCS, and for this reason all previous reports about cAMP induction by SHBG and estradiol were carried out on cells maintained in serum-free medium.

Our data clearly show that estradiol alone is able to increase cAMP in MCF-7 cells in the presence of DCC-FCS, and that this depends on the DCC-FCS concentration used (10% DCC-FCS is effective; 5% DCC-FCS has no effect). The dependence on serum concentration suggests that the estradiol action could be mediated by serum factors whose excessive dilution brings about a loss of the effect. It is interesting to note that the effective dose of estradiol was similar to the dose used by Aronica (1 nM) and also that a significant increase of cAMP was already present after 15 min.

In order to clarify how much SHBG was responsible for mediating estradiol action, DCC-FCS was passed through an affinity column to remove bovine sex hormone-binding globulin (SHBG-FREE/DCC-FCS) and the cAMP induction experiments were repeated. The synergy between estradiol and FCS resulting in cAMP increase was completely lost when bovine sex hormone-binding globulin was removed from serum. In fact, the levels of cAMP observed in MCF-7 cells treated with increasing estradiol concentrations were totally similar to basal levels, even when 10% SHBG-FREE/DCC-FCS was used. When cells were pre-incubated with 50 nM human purified SHBG, estradiol was again able to increase cAMP levels. Therefore, SHBG is one of the serum factors mediating estradiol ability to trigger the cAMP pathway in MCF-7 cells.

The effect of SHBG and estradiol was also evaluated in serum-free medium. We noticed that the needed concentrations of estradiol were different. cAMP induction was detectable with estradiol doses higher than 10 nM in serum-free medium, and lower than 10 nM in 10% SHBG-FREE/DCC-FCS. It is, therefore, likely that other serum factors cooperate to modify the sensitivity of the system. Moreover, only in 10% SHBG-FREE/DCC-FCS, SHBG alone, without addition of steroid hormones, caused a significant increase of cAMP. It could be hypothesized that a portion of estradiol is entrapped in cell membranes, despite the strictly controlled culture conditions, and that 10% SHBG-FREE/DCC-FCS is enough to solubilize and make it available to interact with the SHBG/SHBG-R complex. This hypothesis is further supported by the observation that this effect was detectable at SHBG concentrations higher than 1 nM, but did not have a dose-dependent behavior. Once the triggering concentration for SHBG has been reached, the magnitude of the effect depends on the estradiol amount released from membranes, which is likely to be the same at any tested SHBG concentration.

The crucial role of the estradiol concentration in this system is also suggested by the apparently bizarre behavior of cAMP in cells treated with different SHBG and estradiol doses (in 10% SHBG-FREE/DCC-FCS). The ability to induce cAMP is not related to a specific molar ratio between SHBG and estradiol. The absolute concentration of estradiol, more than the estradiol/SHBG ratio, seems to modulate the effect on the second messenger increase. The peculiar sensitivity of this system could be due to the intricate and successive interactions (first SHBG binds to SHBG-R and then estradiol binds to the SHBG/SHBG-R complex) required to induce cAMP, which could be further modulated by other ''factors'' contained in FCS, as previously suggested.

In conclusion, estradiol can act via the cAMP system in MCF-7 cells as previously suggested [9], but its action is mediated by FCS. SHBG has been demonstrated to be one of the serum factors mediating estradiol membrane action. Moreover, the SHBG/SHBG-R system of MCF-7 cells acts through cAMP, not only in serum-free medium and at very low SHBG doses as already reported [5], but also in a *physiologic* cellular environment (in the presence of serum) and at *physiologic* SHBG concentrations.

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