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Estrogen action via the cAMP signaling pathway: Stimulation of adenylate cyclase and cAMP-regulated gene transcription

(estrogens/antiestrogens/breast cancer/uterus)

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ABSTRACT **Estrogenic hormones, believed to exert most of their effects via the direct interaction of their receptors with chromatin, are found to increase cAMP in target breast cancer and uterine cells in culture and in the intact uterus *in vivo*. Increases in intracellular cAMP are evoked by very low concentrations of estradiol (half maximal at 10 pM) and by other physiologically active estrogens and antiestrogens, but not by an inactive estrogen stereoisomer. These increases in cAMP result from enhanced membrane adenylate cyclase activity by a mechanism that does not involve genomic actions of the hormones (are not blocked by inhibitors of RNA and protein synthesis). The estrogen-stimulated levels of cAMP are sufficient to activate transcription from cAMP response element-containing genes and reporter plasmid constructs. Our findings document a nongenomic action of estrogenic hormones that involves the activation of an important second-messenger signaling system and suggest that estrogen regulation of cAMP may provide an additional mechanism by which this steroid hormone can alter the expression of genes.**

For many years, steroid hormones and peptide hormones have been considered to act via distinctly different mechanisms, the former via intracellular receptors acting through the genome (1, 2) and the latter via membrane-localized receptors that initially affect extranuclear activities, including the generation of second messengers such as cAMP. However, there has been increasing evidence for interactions between cAMP and estrogen in enhancing the growth of the mammary gland and breast cancer cells (3, 4) and for cAMP induction of estrogen-like uterine growth (5). As early as 1967, Szego and Davis (6) demonstrated a very rapid, acute elevation of uterine cAMP by estrogen treatment of rats *in vivo* that was confirmed in other reports (7, 8), but several subsequent studies either failed to confirm this observation or reported only minimal effects that were considered to represent indirect effects of estrogen on cAMP mediated by estrogen-induced release of uterine epinephrine (9–12). Recently, cAMP and other protein kinase activators have been documented to synergize with steroid hormone-occupied receptors, leading to enhanced steroid receptor-mediated transcription (13–18), possibly by a mechanism involving phosphorylation of the receptor or associated transcription factors (14, 19–21).

In this paper, we show that estrogen activates adenylate cyclase, markedly increasing the concentration of cAMP in estrogen-responsive breast cancer and uterine cells in culture and in the intact uterus of rats treated with estrogen *in vivo*, in a manner that does not require new RNA or protein synthesis. The intracellular concentrations of cAMP achieved by low, physiological levels of estrogen are substantial and sufficient to stimulate cAMP response element (CRE)-mediated gene transcription. Therefore, this nongenomic action of the steroid hormone estrogen involves the production of an important second messenger and the resultant activation of second messenger-stimulated genes. These findings document a two-way directionality in the cross talk between steroid hormone- and cAMP-signaling pathways.

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MATERIALS AND METHODS

Plasmid DNA. The SRIF-CAT plasmid (22), containing the CRE region of the somatostatin [somatotropin release-inhibiting factor (SRIF)] gene promoter linked to the gene encoding chloramphenicol acetyltransferase (CAT), was kindly provided by Marc Montminy (Salk Institute, La Jolla, CA). PR_d-CAT, containing the distal promoter of the rat progesterone receptor (PR) gene linked to the CAT gene, has been described (23). pS2-CAT was constructed by replacing the thymidine kinase (tk) promoter of pTZ-tk-CAT (23), released by digestion with *Bam*HI and *Bgl* II, with the promoter region of the gene encoding protein pS2 (−90 to +10; ref. 24), which was released from a genomic clone provided by Pierre Chambon (Institut National de la Santé et de la Recherche Médicale, Strasbourg, France) by *Bam*HI digestion. The PR_d and pS2 promoter regions do not contain any estrogen-response elements (EREs) or CREs and are unresponsive to 17β-estradiol (E_2) and cAMP (refs. 23 and 24 and our unpublished observations). CRE-pS2-CAT and CRE-PR_d-CAT were constructed by annealing the oligonucleotide 5′-TGGCTGACGTCAGAGA-3′ with its complement and cloning the resulting double-stranded oligomer, which contains the CRE of the rat somatostatin gene and its immediate flanking sequences (22) into *Sma* I-digested pS2-CAT and PR_d-CAT, respectively.

Cell Culture, Transfections, and CAT Assays. Primary cultures of uterine cells from 18-day-old Sprague–Dawley rats (Holtzmann, Madison, WI) were prepared and maintained as described (14). MCF-7 human breast cancer cells were maintained as described (23) and were switched to culture medium lacking phenol red 2 days prior to plating for experiments. For cAMP studies, uterine and MCF-7 cells were plated at a density of 1.5–2 × 10^6 cells per 160-mm dish. For transfection experiments, both cell types were plated at 4–5 × 10^6 cells per 100-mm dish, and the medium was changed 48 hr later. Cells were transfected 24 hr later as described (14, 23) with 15 μg of DNA containing 5 μg of CAT reporter plasmid, 3 μg of pCH110 β-galactosidase reporter plasmid, and 7 μg of pTZ19 carrier DNA. Treatments were added in fresh medium following glycerol shock, and cells

Abbreviations: CAT, chloramphenicol acetyltransferase; CRE, cAMP response element; CT, cholera toxin; E_2, 17β-estradiol; ER, estrogen receptor; ERE, estrogen response element; PDE, phosphodiesterase; RIA, radioimmunoassay; TOT, the antiestrogen *trans*-4-hydroxytamoxifen; IBMX, isobutylmethylxanthine; SRIF, somatotropin release-inhibiting factor (i.e., somatostatin); PR, progesterone receptor.

were harvested 24 hr later. CAT assays, normalized for the β-galactosidase activity of each extract, were performed as described (14).

cAMP Radioimmunoassay (RIA). Nearly confluent plates of cells were treated with various agents and were harvested in 500 μl of TNE (40 mM Tris·HCl, pH 7.5/140 mM NaCl/1.5 mM EDTA). Cells were scraped loose using a rubber policeman, collected into microcentrifuge tubes kept on ice, and pelleted (100 × *g* for 5 min). Cell pellets were resuspended in 200 μl of ice-cold cAMP extraction buffer (50 mM Tris·HCl, pH 7.5/4 mM EDTA). For whole uterus treated *in vivo*, the tissue was cut into 1-mm pieces prior to homogenization. Extracts remained on ice for 15 min, with vigorous mixing every 5 min. Extract aliquots were analyzed for protein content by the BCA (bicinchoninic acid) method (Pierce). Extracts were boiled for 10 min, and cell debris was pelleted. A [^{3}H]cAMP RIA kit and instructions (Amersham) were used to measure cAMP content of samples. Incubations were carried out at 4°C for 3 hr, and charcoal–dextran-treated samples were analyzed for radioactivity.

Adenylate Cyclase Assay. Membrane preparations isolated from cultured cells or uteri were tested for adenylate cyclase activity (25). In brief, homogenates in 75 mM Tris·HCl, pH 7.4/2 mM EDTA/8 mM $MgCl_2$ were subjected to two centrifugations (1000 × *g* for 5 min at 4°C and 40,000 × *g* for 20 min at 4°C). Membrane protein (20 μg) was used from each sample. Reaction conditions were as described (25) except that 0.4 μCi (1 μCi = 37 kBq) of [α-^{32}P]ATP was used per tube and the incubation time was extended to 20 min. Reactions were terminated upon addition of 1 mM ATP and 0.1 μCi of [^{3}H]cAMP. Aliquots were assayed for ^{32}P and ^{3}H as a measure of total radioactivity in each sample.

The amount of [^{32}P]ATP converted into cAMP was determined by immunoprecipitation. Precleared samples were incubated with polyclonal antiserum directed against cAMP (provided by V. Ramirez, University of Illinois) for 1 hr on ice. This antiserum elicited no detectable cross-reactivity with ATP, GTP, AMP, or cGMP in our assay system. A second bridging goat anti-rabbit monoclonal antibody was added for an additional 1 hr (Zymed), and antibody complexes were precipitated by using a 10% slurry of Zysorbin (Zymed) as described (14). Zysorbin cells were boiled for 10 min and pelleted. ^{32}P and ^{3}H radioactivity were determined in the supernatant, and recovery of the known [^{3}H]cAMP cpm added (which ranged from 70% to 90%) was used to assess the efficacy of the precipitation and to adjust the ATP-to-cAMP conversion values accordingly. Adenylate cyclase activity is calculated as the percentage of [^{32}P]ATP converted to [^{32}P]cAMP during the 20-min incubation.

Phosphodiesterase (PDE) Assay. The method of Prosser (26), as adapted from Thompson *et al.* (27), was used to assess cAMP PDE activity in cells exposed to various agents. After homogenization in 50 mM Tris·HCl, pH 7.4/1.5 mM EDTA/10% (vol/vol) glycerol/0.6 M NaCl, samples were sonicated for 30 sec on ice (setting no. 3, Branson Sonifier 200) and centrifuged (30,000 × *g* at 4°C for 15 min). Supernatants (20 μg of cell protein) were incubated with 10 μg of snake venom (*Ophiophagus hannah*; Sigma) and 0.15 μCi of [^{3}H]cAMP in assay buffer (4 mM Tris/1 mM 2-mercaptoethanol/1 μM AMP/1 mM $MgCl_2$/0.6 μM cAMP) at 30°C for 15 min. Reactions were stopped by addition of 1 ml of AG 1-X2 resin (Bio-Rad), diluted 1:1 with water, and centrifuged 10 min at 2500 × *g*. The supernatant was collected, added to a second 1-ml aliquot of the resin mixture, and centrifuged a second time. PDE activity of the final supernatant is expressed as pmol of AMP produced from the [^{3}H]cAMP per mg of extract protein per min.

RESULTS

We first examined the effects of estrogen on cAMP in two estrogen-responsive cell types—namely, MCF-7 human breast cancer cells, which contain high levels of endogenous estrogen receptor (ER) and primary cultures of rat uterine cells, which contain lower levels of ER (Fig. 1). In both cell systems in culture, we observed that the estrogen E_2 increased cAMP levels dramatically, as did the antiestrogens *trans*-hydroxytamoxifen (TOT) and ICI 164,384, although the response to antiestrogen was somewhat lower. Maximal, *ca.* 10-fold, increases in intracellular cAMP, monitored by RIA, were observed at 0.5 or 1 hr after exposure to E_2 in MCF-7 and uterine cells, respectively; these represented changes that were one-third to one-half of those achieved by exposure to cholera toxin (CT), a powerful stimulator of adenylate cyclase activity. Intracellular cAMP levels returned to the control level by 6 hr (MCF-7 cells) or 3 hr (uterine cells) despite continued exposure to hormonal ligands. By contrast, cAMP elevation was more prolonged in the presence of CT.

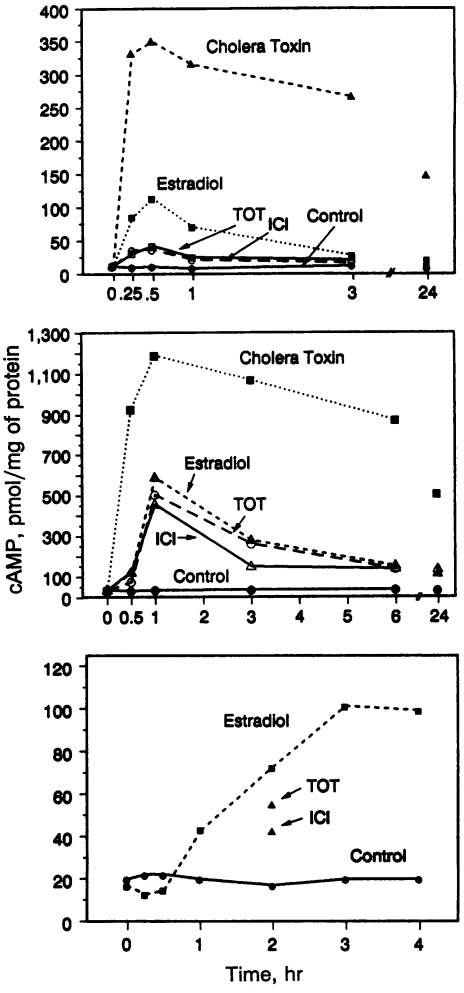

FIG. 1. Effect of estrogen and antiestrogens on the level of cAMP in uterine cells (*Top*), MCF-7 cells (*Middle*), and uterus *in vivo* (*Bottom*). Cultures of immature rat uterine cells (*Top*) or MCF-7 breast cancer cells (*Middle*) were treated with various agents and harvested at the times indicated. Cell extracts were monitored for cAMP content by RIA. Treatments were control vehicle (control), CT at 1 μg/ml, 1 nM E_2, 1 μM TOT, or 1 μM ICI 164,384 (ICI). Each point represents the mean from three experiments. (*Bottom*) Immature 18-day-old rats were given a single s.c. injection of E_2 (5 μg), TOT or ICI (50 μg), or control (0.2 ml of corn oil/ethanol vehicle). Uterine extracts were analyzed for cAMP content by RIA. Each point represents the mean from two experiments.

Exposure of rats to estrogen (E_2) or antiestrogen (TOT, ICI 164,384) *in vivo* also resulted in substantial increases in intracellular uterine cAMP, suggesting that the changes observed *in vitro* are neither artifactual nor associated only with *in vitro* cell culture. Tamoxifen *in vivo* has also been reported to alter cAMP accumulation in quail oviduct and mouse uterus (28).

Dose–response studies reveal that low, physiological concentrations of E_2 were able to stimulate cAMP accumulation (Fig. 2). Half-maximal stimulation was achieved with *ca.* 10 pM E_2 and full stimulation with 1 nM E_2. Slightly higher concentrations of ICI 164,384 and TOT were needed for equal stimulation of intracellular cAMP, consistent with their lower availability in serum-containing medium and their restricted entry into cells. A nearly identical dose–response relationship was observed for cAMP stimulation by these ligands in rat uterine cells (data not shown).

The hormone specificity of the cAMP stimulation was examined in MCF-7 and uterine cells (Table 1). The potent estrogens E_2 and diethylstilbestrol (DES) evoked large fold increases in cAMP, although DES (at 1 nM) was less effective than E_2, while the weaker estrogen 2-hydroxyestrone (2-OH-estrone) effected a substantial but smaller increase in cAMP; the E_2 stereoisomer 17α-estradiol, known to be biologically ineffective as an estrogen, failed to alter intracellular cAMP. The glucocorticoid dexamethasone and the androgen testosterone failed to increase cAMP. As seen in Table 1, nearly identical results were observed in both cell types.

The ability of estrogen to increase cAMP was associated with increased adenylate cyclase activity, with no significant change in PDE activity (Fig. 3). Treatment of cells with CT evoked an increase of *ca.* 3-fold in adenylate cyclase activity within 20 min, while treatment with E_2 or the antiestrogens TOT and ICI 164,384 resulted in increases that were half as great. The effects of E_2, CT, and ICI 164,384 were not additive. Similar increases in uterine adenylate cyclase activity were also observed after treatment of rats with these agents *in vivo* or after treatment of uterine cell cultures *in vitro* (data not shown). By contrast, E_2, TOT, or ICI 164,384 did not alter PDE activity of MCF-7 cells (Fig. 3 *Right*) or uterine cells (data not shown); the potent inhibitor IBMX was effective in suppressing PDE activity, as expected.

To assess whether RNA or protein synthesis was required for the stimulatory effect of these agents on intracellular cAMP concentration, cells were pretreated with actinomycin D or cycloheximide for 30 min prior to the addition of hormone or CT, and cAMP was monitored 1 hr thereafter. Neither of these agents showed any inhibition of the estrogen- or antiestrogen- or CT-induced stimulation of cAMP,

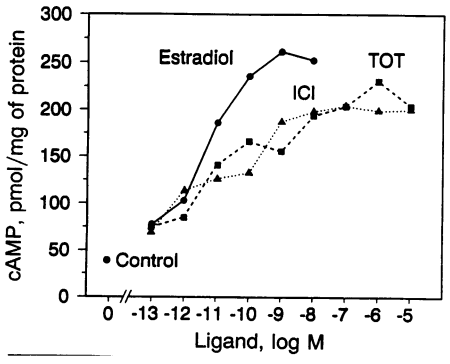

FIG. 2. Dose–response relationship for the increase in cAMP stimulated by estrogen and antiestrogens. MCF-7 cells were treated with various concentrations of E_2, TOT, ICI 164,384 (ICI), or control ethanol vehicle for 1 hr. Cell extracts were monitored for cAMP content by RIA. Each point represents the mean from two experiments.

Table 1. Hormone specificity of the cAMP increase in target cells

Hormone	cAMP level, fold increase over control	
	MCF-7 cells	Uterine cells
E_2	9.2	8.2
DES	5.2	3.8
2-OH-estrone	3.6	3.6
17α-Estradiol	1.1	1.4
Dexamethasone	1.1	1.2
Testosterone	1.0	0.8

Results shown are the mean from two experiments. MCF-7 or uterine cells were treated with various hormones (at 1 nM) for 1 hr or 30 min, respectively, and cell extracts were monitored for cAMP content by RIA. DES, diethylstilbestrol.

although they inhibited RNA or protein synthesis, respectively, by >95% (data not shown). This implies that stimulation of cAMP accumulation does not require RNA or protein synthesis. The very early time course of cAMP increase (Fig. 1) would also be consistent with the apparent absence of a requirement for RNA or protein synthesis.

Since estrogens and antiestrogens increased intracellular cAMP, we asked whether the changes in cAMP were significant enough to affect transcriptional activation of cAMP-regulated genes. To examine this, cells were transfected with a cAMP-responsive gene construct (SRIF-CAT) containing the CRE region of the somatostatin gene promoter (−71 to +55) linked to a CAT reporter gene (22). Cells were treated as shown in Fig. 4, and CAT activity was measured at 24 hr. E_2 exposure evoked a 5-fold (uterine cells) or 13-fold (MCF-7 cells) increase in CRE-mediated CAT activity, nearly equal to that evoked by exposure of cells to 0.1 mM 8-bromo-cAMP. As expected, CT evoked an even larger response based on its greater and more prolonged elevation of cAMP. TOT and ICI 164,384 also stimulated SRIF-CAT activity, although less dramatically than E_2, consistent with their lesser stimulation of intracellular cAMP. The effects of E_2 and CT or cAMP were not additive, consistent with their action via the same pathway. The effects of E_2 plus antiestrogen were also not additive. Insulin-like growth factor 1, which failed to increase intracellular cAMP (data not shown), also failed to increase SRIF-CAT transcription.

The SRIF-CAT cAMP-responsive reporter gene construct contains the somatostatin promoter and flanking region (−71 to +55) and thus might potentially contain elements other than CREs that might contribute to the response to the hormonal agents. Therefore, we prepared two more simple consensus CRE-containing CAT reporter plasmids; the first contained one CRE upstream of the pS2 promoter (−90 to +10) and CAT gene (CRE-pS2-CAT), and the second contained one CRE upstream of the rat PR gene distal promoter and CAT gene (CRE-PR_d-CAT). Transactivation of these reporter genes was stimulated by estrogen and cAMP (Fig. 5), as seen for the SRIF-CAT plasmid, confirming that transactivation of the SRIF-CAT plasmid was indeed mediated via the CRE and, therefore, was due to intracellular cAMP generated by the hormone. The lack of response to E_2 or cAMP in control plasmids (pS2-CAT or PR_d-CAT) containing the pS2 or rat PR promoters and CAT reporter gene but lacking the CRE further confirms the requirement for the CRE in the transactivation.

The possibility of any binding of ER to consensus CRE monomers and dimers was analyzed by standard and competitive gel mobility-shift assays using human ER overexpressed in COS-1 cells (29). In the standard gel mobility-shift assay, direct binding of ER to a radiolabeled CRE dimer could not be detected, whereas binding of bacterially ex-

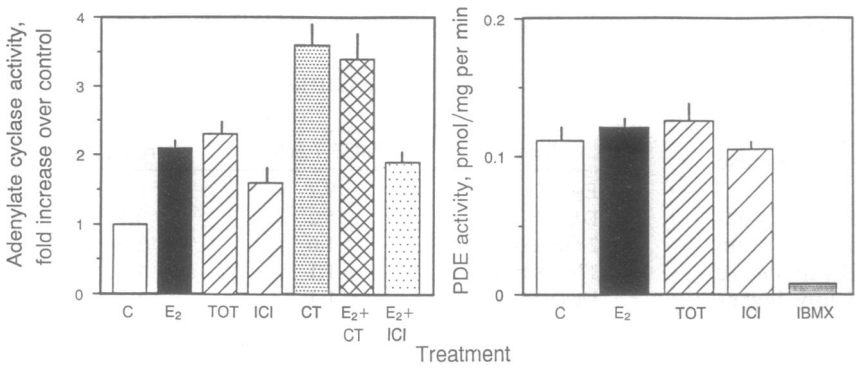

FIG. 3. Regulation of adenylate cyclase and PDE activities by estrogen and antiestrogens. MCF-7 cells were treated for 1 hr with ethanol vehicle (C, control), CT at 1 μg/ml, 1 nM E_2, 1 μM TOT, 1 μM ICI 164,384 (ICI), 0.1 mM isobutylmethylxanthine (IBMX), or E_2 in combination with CT or ICI. Cell extracts were monitored for PDE activity (*Right*), or membrane fractions were monitored for adenylate cyclase activity (*Left*). Each bar represents the mean and range from two experiments. All adenylate cyclase values of treated groups were significantly different from the control ($P < 0.05$ by Student's t test), while only PDE activity of IBMX-treated cells was significantly different from the control.

pressed CREB protein (CRE-binding protein; provided by Richard Goodman, Vollum Institute, Portland, OR) was observed (not shown). In the competitive assay, where the binding of ER to radiolabeled consensus EREs was done in the presence of increasing amounts (up to 400-fold excess) of unlabeled CRE monomer or CRE dimer, no competition at all was observed, suggesting no interaction of ER with the CREs (not shown); however, with unlabeled ERE, a 5-fold molar excess gave nearly complete competition.

DISCUSSION

Three pieces of evidence indicate that the cAMP stimulation by estrogen and antiestrogens is mediated by a high-affinity hormone binder, possibly the ER: first, the steroid specificity and relative effectiveness of different estrogens in stimulation of cAMP (Table 1); second, the low concentrations of E_2 needed for the cAMP stimulation response (Fig. 2), with a half-maximum approximately that of the K_d for E_2 binding to ER; and third, the stimulation by estrogen of increased intracellular cAMP in ER-containing cells with little or no stimulation in cells lacking significant numbers of ERs (such as ER-negative 231 breast cancer cells and 3T3 mouse fibroblast cells, data not presented).

The implications of these observations may be significant. They suggest that estrogens and antiestrogens, by increasing cAMP within target cells, may modulate expression of cAMP-regulated genes and thereby possibly influence other cAMP-regulated bioactivities. Although our studies document an increase in adenylate cyclase activity stimulated by estrogen, the mechanism by which estrogen enhances adenylate cyclase activity remains to be determined. It is of note that almost all responses to estrogen, even early ones such as the induction of creatine kinase B, require RNA synthesis, with some later responses to estrogen also often requiring prior protein synthesis (30, 31). Therefore, the cAMP increase evoked by estrogen is unusual in that prior RNA and protein synthesis are not required. It is also of interest that both estrogens and antiestrogens have stimulatory effects on cellular cAMP levels and enhance CRE-mediated gene transcription. Both categories of ligands also increase ER phosphorylation, and for both end-points, E_2 is somewhat more stimulatory than the antiestrogens (14, 19, 20). By contrast, antiestrogens fail to stimulate, or stimulate only weakly, the transcription of genes containing EREs, which are strongly activated by estrogen (refs. 13, 14, and 23 and references therein).

The clear change in adenylate cyclase activity in response to estrogen suggests a possible membrane action of this hormone, an aspect that will require further study. There is now evidence for ER-like binding sites for estrogens (32) and glucocorticoid receptor-like binding sites for glucocorticoids in the cell membrane (33). Earlier work (34) and more recent studies (35) have provided evidence for ER-like sites in the membrane and cytoplasmic portion of the cell that do not appear to translocate into the nuclear compartment. In the

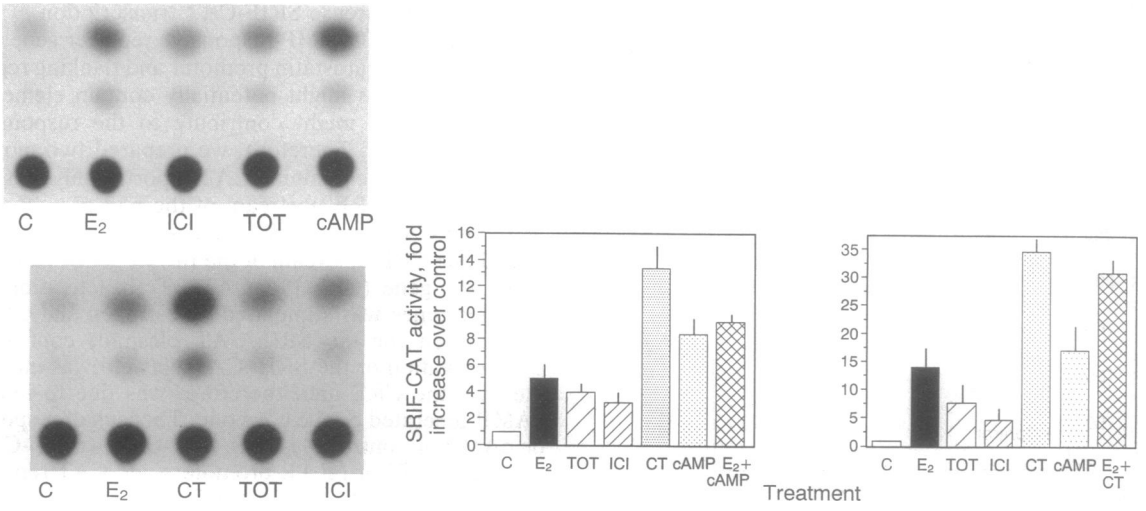

FIG. 4. Induction of CRE-mediated CAT activity by E_2, cAMP, TOT, and ICI 164,384 (ICI). (*Left*) Uterine cells (*Left Upper*) or MCF-7 cells (*Left Lower*) were transfected with the reporter plasmid SRIF-CAT plus an internal control plasmid that expresses β-galactosidase. Treatments were control vehicle (C), 1 nM E_2, 1 μM ICI 164,384 (ICI), 1 μM TOT, 0.1 mM 8-Br-cAMP (cAMP), or CT at 1 μg/ml. Cells were harvested after 24 hr, and extracts were assayed for β-gal activity to normalize for transfection efficiency. Autoradiograms show CAT activity from transfected cells after the indicated treatments. (*Center* and *Right*) Summary of CAT data from uterine cells (*Center*) and MCF-7 cells (*Right*). Bars represent the mean and range from two experiments. All treatment values are significantly different from the control with $P < 0.05$ by Student's t test.

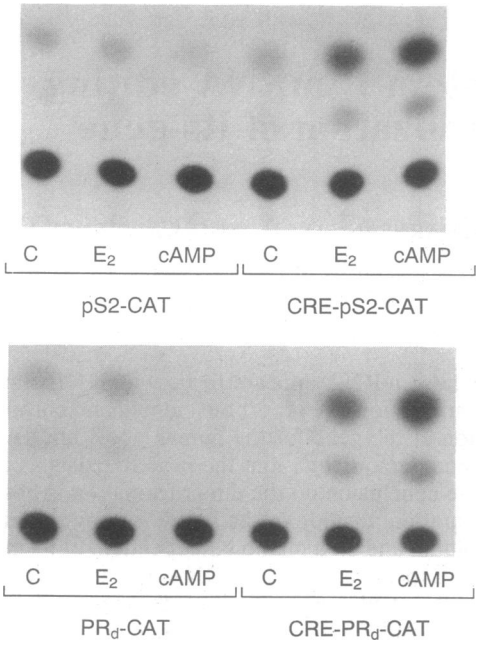

FIG. 5. Stimulation of CAT activity by estrogen and cAMP on target reporter genes containing minimal CREs. (*Upper*) Uterine cells were transfected with plasmid DNA containing either the promoter of the pS2 gene linked to the CAT gene (pS2-CAT) or the pS2-CAT construct containing a single consensus CRE placed upstream of the promoter (CRE-pS2-CAT) plus an internal control plasmid that expresses β-galactosidase. (*Lower*) Uterine cells were transfected with plasmid DNA containing either the distal promoter of the rat PR gene linked to the CAT gene (PR_d-CAT), or the PR_d-CAT construct containing a consensus CRE placed directly upstream from the PR promoter (CRE-PR_d-CAT) plus an internal control plasmid expressing β-galactosidase. Cells were treated for 24 hr with control vehicle (C), 1 nM E_2, or 0.1 mM 8-bromo-cAMP (cAMP). Cell extracts analyzed for CAT activity contained equal amounts of β-galactosidase activity. Autoradiograms show the CAT activity from transfected cells.

case of the steroid hormone progesterone, the evidence is for relatively high-affinity binding sites in the membrane with ligand binding selectivity and other properties clearly different from that of intracellular PRs (36). In addition, several recent reports present clear evidence for other nongenomic effects of E_2 and of vitamin D (37).

Considerable recent data indicate the modulation of steroid hormone receptor action by cAMP and by activation of protein kinase A as well as protein kinase C and tyrosine kinase pathways (13–18, 21, 38, 39). In addition, some growth factors, such as epidermal growth factor and insulin-like growth factor 1, which may act via alterations in signaling pathways involving protein kinases, have been shown to mimic some of the effects of estrogen (14, 40). cAMP has also been shown to be an important modulator of ER bioactivity, able to synergize with estrogen in enhancing ER transcriptional activity (13, 18). Our current observations, plus the reports that vitamin D action increases intracellular cGMP (41), imply a possibly broad involvement of steroid hormone action on cyclic nucleotide and second-messenger systems. Our data provide evidence for extensive two-way cross talk between estrogen and cAMP signaling pathways: in one way as shown previously (13, 14), cAMP can enhance the transcription of estrogen-regulated genes containing EREs; in the other way, as shown here, estrogens can act via the cAMP system to regulate cAMP-mediated gene expression. The consequences of this in terms of understanding the biology and regulation of estrogen-responsive cells seem manifold.

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