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Pretreatment with 17β-Estradiol Attenuates Basal- and PTH-Stimulated Membrane Adenylyl Cyclase Activity in Human Osteoblast-like SAOS-2 Cells

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Parathyroid hormone (PTH)-stimulated production of cAMP by intact human osteoblast-like SaOS-2 cells is diminished by pretreatment with 17β-estradiol (E_2). The goal of the present study was to determine whether E_2 affected adenylyl cyclase activity in cell membranes. Cells were deprived of steroid hormones for 24 h and then incubated with E_2 (1 nM) or vehicle for 12 h. Cell membranes were prepared and incubated with [α-^{32}P]-ATP in the absence or presence of agonist, and the amount of [^{32}P]-cAMP produced was measured to quantify adenylyl cyclase activity. There was less cAMP produced by membranes from E_2-treated cells in response to PTH, GTPγS, forskolin, and Mn^{2+}. E_2 had no effect on the amounts of G_s or G_i. We propose that pretreatment of SaOS-2 cells with E_2 reduced membrane cyclase activity, at least in part, by actions on the PTH-sensitive adenylyl cyclase and/or the G protein-adenylyl cyclase complex.

Past studies have established that estrogen is important in bone metabolism (1, 2). 17β-estradiol prevented PTH-stimulated resorption and prostaglandin production in cultured neonatal mouse calvariae (3). *In vivo* studies also show the protective effects of estrogens against bone resorption stimulated by parathyroid hormone (PTH). Cosman et al. (1) measured the effect of estrogen pretreatment on bone turnover in osteoporotic women who were infused with PTH and found that estrogenized postmenopausal osteoporotic skeletons were less sensitive to the bone resorption-stimulating action of acutely administered PTH than were skeletons of control subjects.

Estrogen receptors have been identified in SaOS-2 cells (4) and in other normal and transformed osteoblast-like cells (5, 6). Fukayama and Tashjian (7) reported that E_2 directly attenuated the PTH-stimulated production of cAMP by intact human osteoblast-like SaOS-2 cells. Other groups have reported similar findings (8). In this paper, we examine the mechanism of action of E_2 on PTH-sensitive adenylyl cyclase in SaOS-2 cell membranes. We report that the action of E_2 was not directed at the PTH receptor and that E_2 attenuated G protein-stimulated catalytic activity of adenylyl cyclase.

MATERIALS AND METHODS

Materials. Dulbecco's Modified Eagle's Medium (DMEM), creatine phosphate, creatine phosphokinase, DNAse I, 17β-estradiol (E_2), NAD^+ and buffer constituents were obtained from Sigma Chemical Co. (St. Louis, MO). Acrylamide, chromatography resins, and b-mercaptoethanol were from Biorad (Richmond, CA). Cholera and pertussis toxins were obtained from List Biological Laboratories (Campbell, CA). Tissue culture plasticware was purchased from Corning (Grand Island, NY). Human PTH_{1-34} (hPTH) was from Penninsula Laboratories (Belmont, CA). ATP and GTPγS were obtained from Boehringer Mannheim (Indianapolis, IN).

Cell culture. Human osteoblast-like SaOS-2 cells (9) were cultured in DMEM supplemented with 5% fetal bovine serum and 5% horse serum. Cells were maintained at 37°C in an atmosphere of 5% CO_2-95% air. 24 h before the start of an experiment, the culture medium was changed to a serum-free, phenol red-free medum to deplete cells of

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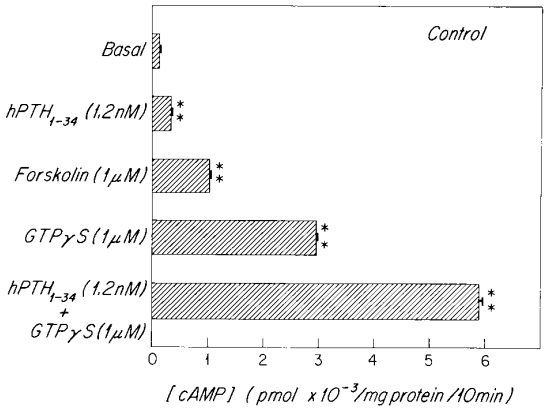

FIG. 1. Basal and agonist-stimulated adenylyl cyclase activity in SaOS-2 Cell Membranes. SaOS-2 cells were grown as described under Materials and Methods without exposure to E_2. Membrane adenylyl cyclase activity was measured and expressed as nmol cAMP per mg protein per 10 min of incubation. Basal activity was measured in the absence of exogenously added agents. Agonists are listed on the ordinate at the concentrations indicated in parentheses.

steroid hormones. E_2 was disolved in ethanol (0.01%) and used at a final concentration of 1 nM. Ethanol vehicle was used as control.

Preparation of membranes. Culture dishes were washed twice with ice-cold homogenization buffer consisting of 10 mM Tris and 0.5 mM EGTA plus 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM dithiothreitol (DTT) at pH 7.8. Cells were harvested with a rubber policeman. Broken cells were disrupted in a bath sonicator (E/MC ultrasonic cleaner model #250) with ten 3-sec pulses. Membranes were isolated by centrifugation at 15,000 $\times$ g for 30 min at 4°C. Protein concentration was determined by the Lowry method (10).

Adenylyl cyclase assay. The assay was performed in buffer which contained: (in mM) 2.5 ATP, 12.5 $MgCl_2$, 50 cAMP, 25 TrisCl, 2.5 EDTA, 2.5 β-mercaptoethanol, 50,000 cpm [^{3}H]-cAMP, plus 0.03–3.0 pmol [α-^{32}P]-ATP (SA ranged from 30–300 cpm/pmol), together with 200 mM creatine phosphate and 3500 U creatine phosphokinase. Control buffer solution consisted of 5 mM papaverine and 5 mg/ml bovine albumin in homogenization buffer. Experiments were initiated by the addition of membranes to the assay mixture and incubation was performed for 10 min at 37°C. The reaction was stopped with a solution of 40 mM ATP, 13 mM cAMP and 69 mM sodium dodecyl sulfate (SDS) followed by heating for 3 min at 100°C. The newly synthesized [^{32}P]-cAMP corrected for recovery by determination of [^{3}H]-cAMP, was isolated chromatographically by passing the reaction mixture sequentially over Dowex AG 50 W X8 and Alumina-Neutral WN-3 columns, and measurement in a liquid scintillation counter.

ADP-ribosylation assay. Cholera and pertussis toxins were activated by incubation in 25 mM DTT for 30 min at 37°C. Membrane proteins were diluted (final concentrations of 1–5 mg/ml) in 75 mM Tris-HCl plus 0.12 mg/ml DNAse I. A solution of 60 μM NAD^+ containing 20 $\times$ 10^6 cpm of [^{32}P]-NAD was prepared. The ADP-ribosylation reaction was performed in (in mM) 600 K_2PO_4, 20 thymidine, 2 ATP, 0.2 GTP, 20 $MgCl_2$, 2 EDTA, and with or without 100 ADP-ribose, pH 7.0, for 45 min at 32°C. The reaction was stopped using 20% trichloroacetic acid (TCA). After a 20-min incubation on ice, the mixture was centrifuged at 2000 rpm in a clinical centrifuge, the supernatant discarded, and the pellet washed with ethyl ether. The pellets were resuspended in buffer consisting of (in %) 40 glycerol, 2 β-mercaptoethanol, 1 SDS, 1.5 Tris, pH 6.8 and then analyzed on 10% SDS polyacrylamide gels (11).

RESULTS

Adenylyl cyclase activity in SaOS-2 cell membranes. The data in Figure 1 show a modest, but significant ($p < 0.01$), enhancement of adenylyl cyclase activity by 1.2 nM hPTH. Forskolin, a direct activator of adenylyl cyclase, also enhanced membrane cyclase activity. The nonhydrolyzable GTP analog, GTPγS, gave a somewhat greater enhancement of cyclase activity. Potentiation of the PTH effect was observed with GTPγS together with hPTH. We conclude that the components of the PTH receptor-G protein-adenylyl cyclase signal transduction pathway are present and functional in this membrane preparation.

Basal adenylyl cyclase activity and agonist-stimulated adenylyl cyclase activity were reduced

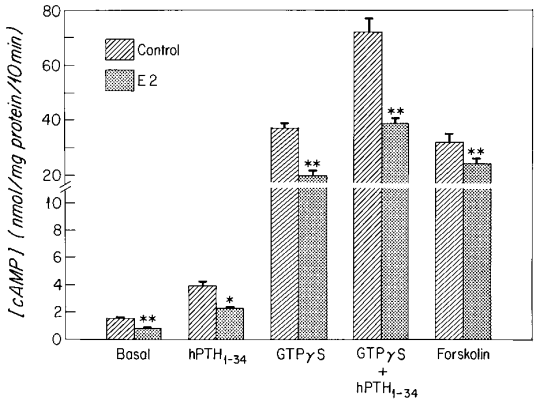

FIG. 2. Adenylyl cyclase activity was reduced in membranes of E_2-treated cells. SaOS-2 cells were depleted of steroid hormones for 24 h and then incubated in the presence or absence of E_2 for 12 h. Membrane adenylyl cyclase activity was measured. Cross-hatched bars give the vehicle-treated controls and stippled bars are the result for E_2 treated cells. Concentrations of agonists were those indicated in Fig. 1. The statistical method used was the paired Student's t test (* for $p<0.05$, ** for $p<0.01$).

in membranes from E_2-treated cells. The data in Figure 2A show that membranes from E_2-treated (12 h) cells generated less cAMP in response to several of agonists than did control membranes. Basal cyclase activity was reduced by 42%, PTH-stimulated activity was reduced by 44%, GTPγS-stimulated activity by 47%, forskolin-stimulated activity by 24%, and hPTH together with GTPγS was reduced by 46%. We conclude that pretreatment of SaOS-2 cells with E_2 attenuated a PTH-sensitive adenylyl cyclase activity. The inhibition of GTPγS-stimulated cyclase activity by E_2 pretreatment suggested that E_2 affected events in the signal transduction pathway occuring distal to the PTH receptor.

Catalytic activity of adenylyl cyclase was attenuated in membranes of E_2-treated cells. Results with forskolin support the view that E_2 acts to affect the catalytic activity of adenylyl cyclase. However, G proteins may play a role in the actions of forskolin (12); therefore, we tested whether direct stimulation of adenylyl cyclase by Mn^{2+} was altered by E_2 pretreatment. Figure 3 shows that Mn^{2+}-stimulated cyclase activity was attenuated in membranes from E_2

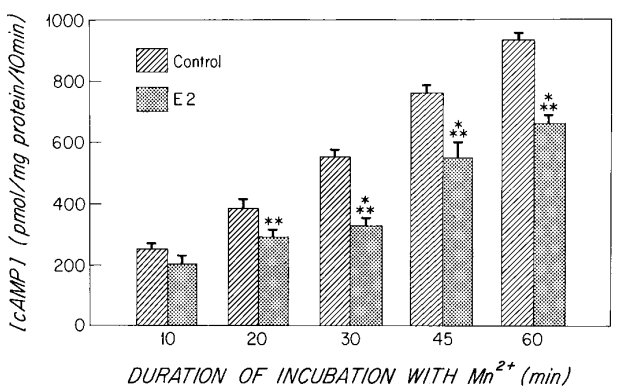

FIG. 3. E_2-pretreatment attenuated direct stimulation of the adenylyl cyclase by Mn^{2+}. Membranes were prepared from E_2- and vehicle-treated cells. Adenylyl cyclase in membranes was stimulated for varying lengths of time by exposure to 10 mM Mn^{2+}. Cross-hatched bars are the result for membranes from vehicle-treated controls and stippled bars are the result for E_2-treated cells. * for $p<0.05$, ** for $p<0.01$

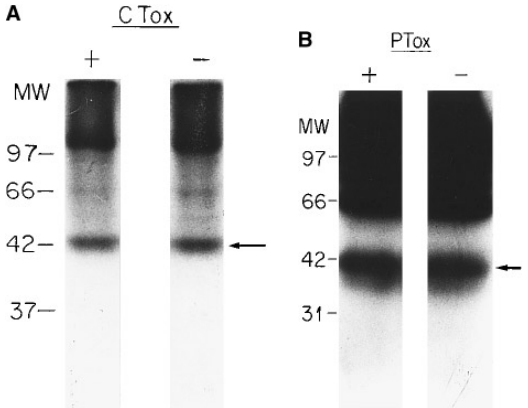

FIG. 4. Membranes from E_2-pretreated cells did not contain reduced levels of G_s and G_i. Membranes were treated without (−) or with (+) cholera or pertussis toxin in the presence of [^{32}P]-NAD$^+$. Labelled proteins were resolved by SDS-PAGE. Arrows indicates the labeled bands with molecular weight corresponding to G_s and G_i. (A) Cholera toxin-treated membranes. (B) Pertussis toxin-treated membranes.

treated cells. The magnitude of the inhibition by E_2 was dependent on the length of time of stimulation by Mn^{2+}. After 20 min of exposure to Mn^{2+}, there was modest inhibition but, after 60 min of incubation with Mn^{2+} adenylyl cyclase activity was inhibited by about 30%. In some experiments the magnitude of inhibition was as great as 70%. We conclude that pretreatment of SaOS-2 cells with E_2 has an inhibitory effect on the catalytic activity of adenylyl cyclase.

E_2 pretreatment did not reduce the amounts of G_s or G_i in cell membranes. An additional possible mechanism for reduction in the production of cAMP by E_2 pretreatment could be a decrease in the amount of the stimulatory G protein (G_s) or increased the amount of the inhibitory G protein (G_i). The amounts of G protein were assessed by labeling G_s or G_i in membranes, without or with E_2 pretreatment, by ADP ribosylation with cholera or pertussis toxin. Results in Figure 4A show that a cholera toxin-labeled band migrates at the expected (42 kDa) position of $G_s\alpha$ and that the amount of labeled protein was essentially unchanged by E_2 pretreatment. We conclude that E_2 had no effect on the amount of G_s in membranes. Pertussis toxin ADP-ribosylates a peptide of 40,000−41,000 kDa (13). Results in Figure 4B show that a pertussis toxin-labeled band migrates at the expected position and that the amount of labeled protein was unchanged by E_2 pretreatment.

E_2 added directly to membranes did not reduce basal or agonist-stimulated adenylyl cyclase activity. We could rule out the classical pathway of E_2 action via regulation of gene transcription if E_2 had a direct effect on isolated cell membranes. We tested the effect of direct addition of E_2 on the adenylyl cyclase assay. Figure 5 shows that there was no inhibitory effect of E_2 on Mn^{2+}-stimulated activity. Basal and PTH-stimulated adenylyl cyclase activity were enhanced by 58 % and 67%, respectively, by direct addition of E_2 to SaOS-2 cell membranes. We conclude that the inhibitory action of E_2 on adenylyl cyclase required intact cells. The stimulatory action of E_2 addition directly on membranes is analogous to enhancement of cAMP accumulation induced by brief treatment of MCF-7 and rat uterine cells with estradiol (14).

DISCUSSION

Action of E_2 was specific for the PTH receptor-sensitive adenylyl cyclase. The attenuating effect of E_2 on agonist-induced activity was on a PTH-sensitive adenylyl cyclase. We conclude that E_2 probably did not act on the PTH receptor because cAMP production stimulated by

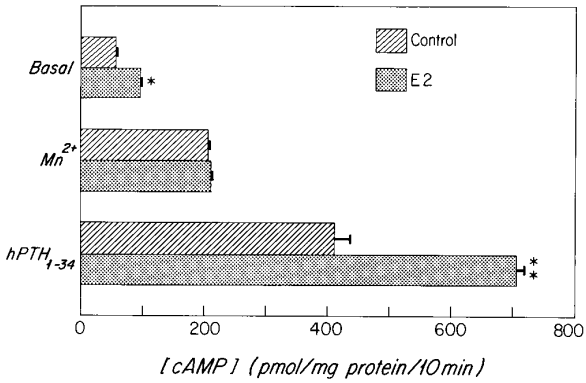

FIG. 5. Direct addition of E_2 to membranes did not attenuate adenylyl cyclase activity. Cells were not preincubated with the steroid hormone instead E_2 (1 nM) was added directly to membranes prepared from steroid depleted cells. Basal (no agonist), Mn^{2+} (10 mM), and PTH (1 nM) stimulated cyclase activities were measured. Cross-hatched bars are the result from vehicle-treated controls and stippled bars are the result from samples containing E_2.

GTPγS, forskolin, and Mn^{2+} was also affected. Binding studies to measure the effect of E_2 on the affinity of PTH for its receptor would confirm this hypothesis.

G protein as a potential target of E_2 action. Previous studies have shown that E_2 pretreatment can increase the amount of G_s in membranes and thus enhance cyclase activity (15, 16). We conclude from the ADP ribosylation experiments that the amounts of $G_s\alpha$ and $G_i\alpha$ in SaOS-2 were not substantially changed by pretreatment of the cells with E_2. A limitation of this method of detecting $G_s\alpha$ and $G_i\alpha$ is that multiple isoforms of $G_s\alpha$ and $G_i\alpha$ which are unrelated to the PTH-adenylyl cyclase transduction pathway may also be labeled. Prolonged E_2 treatment ($>$ 12 h) could affect G protein function, without altering mass, for example, by a reduction in the rate of GTP hydrolysis by G_s, an enhancement in the affinity of G_s for GDP, or enhancement of coupling between G_i and adenylyl cyclase. It has been shown that acute (within 5 min) E_2 treatment of pituitary cell plasma membranes can enhance the GTPase activity of G_s which could explain the attenuation of of receptor-stimulated cyclase activity by E_2 in SaOS-2 cells.

Inhibition of the catalytic activity of adenylyl cyclase by E_2. E_2 pretreatment led to a reduction in cAMP production stimulated by either forskolin or Mn^{2+} suggesting that E_2 reduced the catalytic activity of adenylyl cyclase. Forskolin is widely considered to be a direct activator of adenylyl cyclase (17); however, forskolin activation of cyclase may also require G_s, possibly by its action to stabilize the G_s-cyclase complex (12). Inhibition by E_2 pretreatment, of the catalytic activity of adenylyl cyclase could explain the reduced basal cyclase activity. Possible mechanisms of E_2 action on the cyclase enzyme include reduction in the affinity of the enzyme for ATP or a reduced amount of the PTH-sensitive cyclase isoform.

The inhibitory action of E_2 required intact cells. Use of the membrane cyclase assay allowed us to explore the possibility that the action of E_2 might be receptor- or nucleus- independent by testing the effect of adding E_2 directly into the assay reaction mixture. A potential criticism of the negative outcome of this experiment is that a 10-min incubation of membranes with E_2 was not sufficient to obtain the inhibitory effect. Aronica et al. (14) showed that estrogen enhanced cAMP accumulation in rat uterine cells, MCF-7 cells, and in the uterus *in vivo;* like the effect in SaOS-2 cell membranes, this stimulatory effect on cAMP levels was rapid (within 15 min). They also reported enhancement of cyclase activity in membranes prepared from cells treated with estrogen for 1 h, effects did not require RNA or protein synthesis (14).

Receptor-like plasma membrane binding sites for estrogens, which do not translocate to the nucleus, have been described (18).

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