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Estrogen Modulates α_1/β-Adrenoceptor-Induced Signaling and Melatonin Production in Female Rat Pinealocytes

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Pineal gland · Gonadal steroids · Gonadal steroid receptors · Catecholamines · Catecholamine receptors · Cyclic AMP · Melatonin · Inositolphosphate

Abstract
Nocturnal rise in pineal melatonin output is due to the night-induced acceleration of noradrenergic transmission and α_1- and β-adrenoceptor activation. In addition, in female animals, cyclic oscillations in circulating levels of sex steroid hormones are accompanied by changes in the rate of pineal melatonin secretion. To investigate whether estrogen directly affects pineal adrenoceptor responsiveness, pinealocytes from 21-day-old ovariectomized rats were exposed to physiological concentrations of 17β-estradiol (17β-E_2) and treated with noradrenergic agonists. Direct exposure to 17β-E_2 reduced α_1/β-adrenoceptor-induced stimulation of melatonin synthesis and release. This effect was mediated by an estrogen-dependent inhibition of both β-adrenoceptor-induced accumulation of cAMP and α_1-adrenoceptor-induced phosphoinositide hydrolysis. Furthermore, estrogen reduced transient Ca^{2+} signals elicited in single pinealocytes by α_1-adrenoceptor activation or by potassium-induced depolarization. In the case of β-adrenoceptor responsiveness, neither forskolin- nor cholera toxin-induced accumulation of cAMP were affected by previous exposure to 17β-E_2. This indicates that estrogen effects must be exerted upstream from adenylylcyclase activation, and independent of modifications in G protein expression, therefore suggesting changes in either adrenoceptor expression or receptor-effector coupling mechanisms. Since estrogen effects upon adrenoceptor responsiveness in pineal cells was not mimicked by 17β-E_2 coupled to bovine serum albumin and showed a latency of 48 h, this effect could be compatible with a genomic action mechanism. This is also consistent with the presence of two estrogen receptor proteins, α- and β-subtypes, in female rat pinealocytes under the present experimental conditions.

In most species, environmental oscillations in lighting conditions are transduced by pineal cells from neuronal to hormonal messages through the rhythmic secretion of melatonin [1]. This process is accelerated during the night by the enhanced release of norepinephrine from sympa-

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thetic nerve terminals arising from the superior cervical ganglion. Activation of pinealocyte β-adrenoceptors by norepinephrine stimulates adenylate cyclase (AC) activity via a Gs protein-dependent mechanism leading to a rapid increase in cAMP levels [2], activation of cAMP-dependent protein kinase, and phosphorylation of cAMP response element binding protein (CREB) [3]. The interaction of phosphorylated CREB protein with a cAMP-response element induces the expression of N-acetyltransferase (NAT), the enzyme that catalyses the rate-limiting step in melatonin synthesis [4]. Phosphorylated CREB also activates an alternative promoter and the expression of an inducible cAMP early repressor, which represses cAMP-induced NAT transcription and also that of its own promotor [5]. Moreover, in pinealocytes, β-adrenoceptor response to norepinephrine is potentiated by coactivation of α_1-adrenoceptors. This results in increased phospholipase C (PLC) activity and phosphoinositide (PI) hydrolysis [6], which in turn raises intracellular Ca^{2+} levels [7] and protein kinase C (PKC) activity [8].

In addition to environmental light, changes in circulating levels of gonadal steroids also influence pineal melatonin output [9, 10]. In female rats, both melatonin synthesis and release are reduced during the night of proestrus [11, 12]. Treatment with ovarian hormones block the β-adrenoceptor-induced rise of pineal melatonin in ovariectomized rats in vivo [13], and regulate the sensitivity of pinealocytes to in vitro adrenergic stimulation [14]. In the present study, we have investigated whether direct exposure of female rat pinealocytes to physiological estrogen concentrations affects melatonin synthesis and release in response to adrenergic stimulation. In addition, direct estrogen effects on β-adrenoceptor-induced cAMP production and α_1-adrenoceptor-induced PI hydrolysis and transient Ca^{2+} signals were also studied.

Materials and Methods

Chemicals

Most chemical reagents, pharmacological agonists, and steroid hormones were purchased from Sigma-Aldrich (Madrid, Spain). Materials from other sources are specified in the text when needed.

Animals

Twenty-one-day-old female Sprague-Dawley rats, housed in a temperature-controlled room (23 ± 2 °C) under a light-dark cycle of 12:12 (60–80 μWcm^{-2}), were bilaterally ovariectomized under ketamine-xylazine anesthesia 48 h prior to use. Animals were sacrificed by decapitation 1 h before lights off, and their pineal glands were quickly dissected out and processed as described below.

Cell Culture and Hormone Treatment

Pineal cells were dispersed by trypsin and DNA-ase treatment as described before [15]. Pinealocytes (200,000 cells ml^{-1}) were plated onto 35 × 15 mm culture dishes (Corning, N.Y., USA) precoated with poly-*D*-lysine. They were incubated at 37 °C for 24 h under humidified atmosphere (95% air, 5% CO_2) in phenol red-free DMEM (Life Technologies, Barcelona, Spain), containing fetal bovine serum (10%), *L*-glutamine (1%), penicillin G (10,000 U/l), and streptomycin (75,000 U/l). Prior to treatment with estrogen, the culture medium was changed to the same DMEM, except that the fetal bovine serum added was steroid-free (dextran-charcoal-stripped fetal bovine serum). Pinealocytes were exposed to 17β-estradiol (17β-E_2, 1 n*M*) or the vehicle (0.001% ethanol) for 48 h (24 h × 2), and then treated with adrenergic agonists as described below.

NAT Activity in Pineal Cells

NAT activity was measured in pinealocytes that had been treated with isoproterenol (ISO, 1 μ*M*) alone, or with ISO in combination with phenylephrine (PHE, 1 μ*M*) for 6 h. Total amount of adrenergic agonists was dissolved in 10 μl of water, and the same volume of this vehicle was added to control cells (CO). The cells were collected and homogenized by sonication (50 W, 15 s) in 200 μl of 0.25 *M* sucrose. NAT activity was assayed by measuring the amount of N-acetyltryptamine formed from acetyl CoA and tryptamine, by high-performance liquid chromatography (HPLC) and fluorometric detection [15]. Aliquots of 50 μl homogenate were mixed with 25 μl of 8 m*M* tryptamine and 25 μl of 4 m*M* acetyl CoA in 0.1 *M* phosphate buffer, pH 6.5, containing 5 m*M* EGTA. Samples were incubated at 37 °C for 30 min and the enzymatic reaction was stopped by addition of 1 ml of ice-cold toluene:isoamyl alcohol (99:1 vol/vol). After shaken for 5 min, samples were centrifuged at 9,000 *g* for 5 min, and 0.5-ml aliquots from the organic phase were taken to dryness. The residue was redissolved in 100 μl of the mobile phase and aliquots of 25 μl were injected into the chromatographic system.

Melatonin in the Incubation Medium

Melatonin content from the culture medium was assayed by HPLC and fluorometric detection as described previously [16]. Aliquots of 500 μl were mixed with 1 ml of chloroform, shaken for 15 min and centrifuged at 9,000 *g* for 10 min. After washing the organic phase twice with 0.05 *M* carbonate buffer, pH 10.25, aliquots of 0.5 ml were taken to dryness. The residue was redissolved in 50 μl of the mobile phase and 30 μl were injected into the chromatographic system.

Chromatographic Procedure

Aliquots prepared as described above were injected onto a C-18 Spherisorb ODS reversed-phase column (particle size 5 μm, Scharlau SA, Barcelona, Spain). For N-acetyltryptamine determination (NAT activity from pineal cells), the mobile phase consisted of 0.1 *M* sodium acetate, 50 mg/l EDTA, and 15% acetonitrile (vol/vol) in distilled-deionized water (Millipore Q System, Millipore Ibérica SA, Barcelona, Spain). The mobile phase was adjusted to pH 5.2, filtered, and continuously degassed with He (20 ml/min). For the determination of melatonin (extracted from the incubation medium), we used the same chromatographic system with a different mobile phase consisting of 0.1 *M* sodium phosphate, 50 mg/l EDTA, and 20% acetonitrile, pH 5. The system was run at a flow rate of 1 ml/min (Waters 600E pump, Millipore Ibérica). The fluorescence detector (LS 40, Perkin Elmer Ltd., Buckinghamshire, UK) was set at excitation/

emission wavelengths of 285/360 nm and 285/345 mm, for the detection of N-acetyltryptamine and melatonin, respectively [15, 16]. The identification of peaks by retention time and their quantification by peak height was done using a HP 3396 integrator (Hewlett Packard Co., Calif., USA). The detection limit was 250 fmol and the inter- and intra-assay variation coefficients were smaller than 7 and 4%, respectively.

cAMP Accumulation

To estimate the effect of α_1- and β-adrenoceptor activation on AC, cAMP accumulation after agonist treatment was measured in cultured pinealocytes. Plated cells were preincubated with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX, 0.1 m*M*) for 60 min, and then they were incubated with ISO (1 μ*M*) alone or in combination with PHE (1 μ*M*) for 15 min. Total amount of adrenergic agonists was dissolved in 10 μl of water, and the same volume of this vehicle was added to control cells (CO). In other experiments, pineal cells were incubated with forskolin (up to 100 μ*M*) or cholera toxin (CTx, 10 μ*M*) for 20 or 30 min, respectively. The cells were scraped from the plates, homogenized by sonication (100 W, 5 s) in 200 μl of 50 m*M* acetate buffer, pH 4.0, and incubated in a water bath at 90°C for 3 min. The cells were then frozen at –80°C until assayed. Cyclic AMP content was determined in duplicate after acetylation, using an enzyme immunoassay kit (EIA, RPN225, Amersham, UK). The detection limit was 12 fmol, and the inter- and intra-assay variation coefficients were smaller than 10 and 7%, respectively.

PI Hydrolysis

To evaluate the effect of pinealocyte α_1-adrenoceptor activation on PI hydrolysis, we measured the total accumulation of labeled inositolphosphates (IP) following stimulation with PHE. Prior to treatment with agonists, the cells were incubated for 12 h with 20 μ*M* (3 μCi/plate) *myo*-[^{3}H]inositol (17.1 Ci/mmol, Amersham, UK) in 2 ml of the growth medium. Pinealocytes were exposed to 1 μ*M* PHE for 1 h, in the presence of 10 m*M* LiCl to prevent [^{3}H]IP degradation by phosphatase activity. At the end of the incubation period the cells were washed twice with phosphate-buffered saline at 37°C, and the reaction was stopped by adding 1 ml of ice-cold methanol. The cells were scraped from the dishes and [^{3}H]IP were extracted as previously described [17]. Briefly, water-soluble metabolites were separated from phospholipids by extraction in methanol-chloroform water (1:1:0.5, v/v/v). [^{3}H]IP were isolated by separating the aqueous phase on anion exchange columns (AG 1-X8, formate form, Bio-Rad Laboratories, Madrid, Spain). After loading the samples, free [^{3}H]inositol was washed off with 6 ml of water and the [^{3}H]IP fraction was eluted from the columns by adding 5 × 1 ml of 1 *M* ammonium formate plus 0.1 *M* formic acid [18]. Radioactivity was measured in aliquots of the fractions by liquid scintillation spectrometry (counting efficiency, 30–40%; Beckman LS380, Calif., USA).

Ca^{2+} Measurements

Determination of intracellular Ca^{2+} ($[Ca^{2+}]_i$) in single cells was done using the membrane-permeable form of the Ca^{2+} indicator dye Fura-2 (Fura-2 acetomethyl ester, Fura 2/AM, Molecular Probes Inc., OM) [19]. For this purpose, pineal cells were cultured as described above onto poly-*D*-lysine-coated coverslips placed into culture dishes. Thirty minutes prior to experiments, pinealocytes were incubated with 2 μ*M* Fura 2/AM at 37°C for 15 min, and subsequently washed in Fura-2-free solution for a minimum of 10 min. The cells were then positioned an a specially prepared chamber on an inverted microscope. Ringer and test solutions were continuously applied by superfusion. The composition of the solution used was (in m*M*): NaCl, 140; KCl, 2.7; $MgCl_2$, 1; $CaCl_2$, 10; HEPES, 10; glucose, 10, and ascorbic acid, 5; pH 7.25–7.35 (NaOH). The test solution containing 1 μ*M* PHE was applied for 15 s, with a dead time of 5–10 s. In some experiments we used a depolarizing test solution by increasing the concentration of KCl to 60 m*M* and decreasing NaCl to 80 m*M* in order to maintain osmolarity. The optic system was composed of an inverted microscope (Axiovert 35, Zeiss, Germany) equipped with a 40 × 0.75 NA Plan neofluor objective. The light from a xenon arc lamp (Osram XB075 W/2) was alternately filtered by means of a rotating wheel at 375 and 390 nm (SWP 357, BP 380/10, from Optisk Laboratories, Lyngby, Denmark). The filtered light was guided through an FT 430-nm dichroic mirror, and its emission passed through an LP 470-nm filter before projection onto the photomultiplier tube. $[Ca^{+2}]_i$ was calculated on-line from the ratio of the emission at 357 nm excitation to that at 390 nm [19].

Western Blotting

Primary cultures of pineal cells were rinsed, harvested (2×10^5/dish), and homogenized with a Dounce homogenizer in 0.2 ml lysis buffer containing 1% (w/w) tergitol (type NP-40), 0.25% (w/v) sodium deoxycholate, 1 m*M* sodium vanadate, 150 m*M* NaCl, 1 m*M* EGTA, 1 m*M* NaF, 1 m*M* phenylmethylsulfonyl fluoride, 50 m*M* Tris-HCl plus 1 μg/ml leupeptin and 5 μg/ml pepstatin A. After centrifugation (12,000 *g*) at 4°C for 10 min, the supernatant was collected and the pellet discarded. Samples containing 40 μg of protein were analyzed by electrophoresis on 11% polyacrylamide sodium dodecyl sulfate gradient gel, and transferred to a nitrocellulose membrane (Immobilon-P PVDF, Millipore, Watford, UK). The membranes were blocked with 3% dried milk fat powder in phosphate-buffered saline for 1.5 h, and blots were then incubated with primary antibodies against estrogen receptor α (ERα, 1:500 dilution; Stressgen Biotechnologies Corp., Victoria, B.C., Canada) and estrogen receptor β (ERβ, 1:500 dilution; Upstate Biotechnology, N.Y., USA). An ECL+ kit (Amershan, Arlington Heights, Ill., USA) was used to visualize the products.

Statistics

Statistical analysis was performed using Statistix 4.1 for PC (Analytical Software, Tallahassee, Fla., USA). Results were analyzed according to a 2 × R (row) factorial design, one factor having two levels (vehicle and 17-E_2), and the other factor (R) having either two (vehicle and ISO, or vehicle and PHE) or three levels (vehicle, ISO, and ISO plus PHE). Some experiments were repeated several times with cells from different animals under the same conditions, and therefore each replication can be considered as one level of an additional factor. In order to evaluate the effect of different treatments for each series of experiments, group means were compared by one-way ANOVA [20], followed by previously designed comparisons. Whenever interaction among main factors was found relevant, the effect of each factor was tested separately for each level of the others. When necessary, correlation and linear regression analysis between two measured variables were also performed.

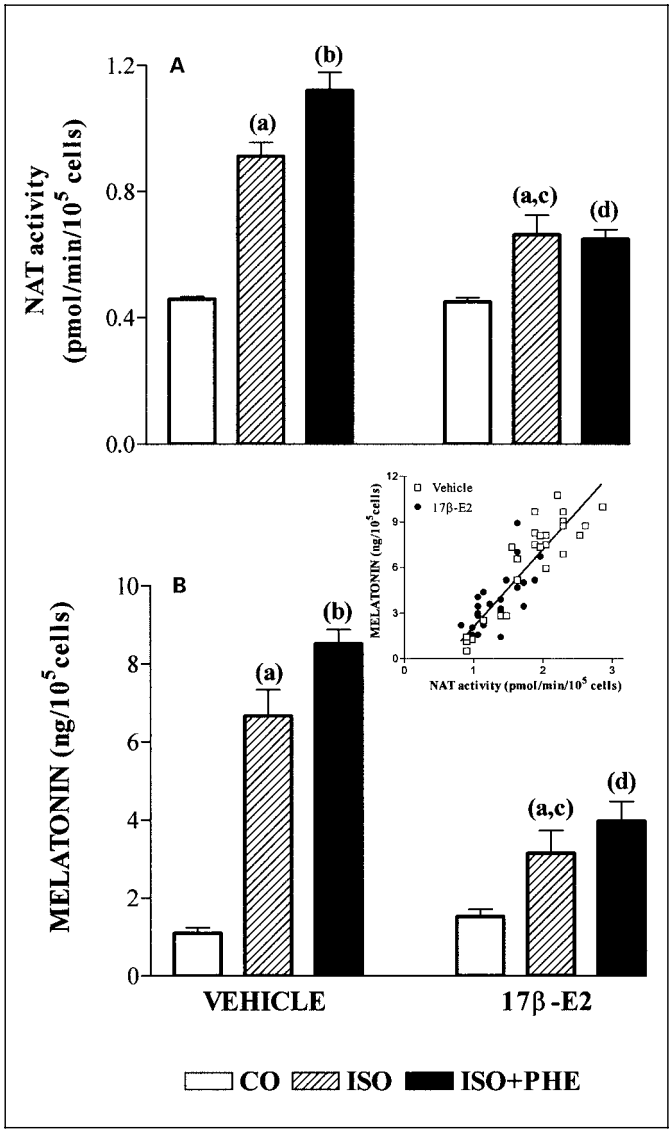

Fig. 1. Effect of 17β-E_2 on α_1/β-adrenoceptor-induced stimulation of NAT activity (**A**) and melatonin release (**B**) in dispersed pinealocytes. Pineal cells from 21-day-old ovariectomized rats were exposed to 17β-E_2 (1 n*M*, 48 h) or the vehicle (0.001% ethanol), as described in the 'Materials and Methods' section. Thereafter, they were incubated for 6 h with ISO (1 μ*M*) or ISO plus PHE (1 μ*M* each). These drugs were added dissolved in 10 μl of water, and the same volume of this vehicle was added to control cells (CO). Pinealocyte NAT activity and melatonin released into the incubation medium were assayed by HPLC with fluorometric detection. The data represent the mean ± SEM of 10–15 plates (in this and other figures each plate contained 200,000 cells ml^{-1}). The inset represents the linear relationship between NAT activity and melatonin release ($r = 0.81$, $p < 0.001$). [a] $p < 0.001$ vs. CO; [b] $p < 0.05$ vs. ISO; [c] $p < 0.01$ vs. vehicle; [d] $p < 0.001$ vs. vehicle.

Results

Effects of 17β-E_2 on α_1/β-Adrenoceptor-Induced Stimulation of NAT Activity and Melatonin Release in Female Rat Pinealocytes

Stimulation of pinealocyte β-adrenoceptors increased NAT activity and the total amount of melatonin released over a 6-hour period, after the addition of ISO (1 μ*M*) to cultured pineal cells (fig. 1). This response was potentiated by the simultaneous activation of α_1-adrenoceptors following addition of PHE (1 μ*M*) to the medium (fig. 1). PHE, alone, had no effect (data not shown). However, incubation of cells with 17β-E_2 (1 n*M*) for 48 h prior to adrenoceptor stimulation reduced the ISO-induced elevation of NAT activity and melatonin release by ~26 and ~53%, respectively, and blocked the potentiation effect of PHE on both parameters (fig. 1). To estimate the relationship between pinealocyte NAT activity and the amount of melatonin released into the medium (as independent and dependent variables, respectively), correlation and linear regression analysis were performed. The levels of NAT activity and the amount of melatonin secreted over a 6-hour period were well correlated (fig. 1, inset; $r = 0.81$, $p < 0.001$), indicating that α_1/β-adrenoceptor-induced elevation of melatonin secretion was mainly a consequence of stimulation of its synthesis. The linear relationship between NAT activity and melatonin levels was not affected by estrogen treatment, since the slopes from vehicle- and 17β-E_2-treated plates were extremely similar, and therefore only one line is represented in the figure (fig. 1, inset).

Estrogen Modulation of Adrenoceptor-Induced Intracellular Signals in Female Rat Pinealocytes

Accumulation of cAMP after α_1/β-Adrenoceptor Stimulation. Since adrenoceptor-induced stimulation of AC and accumulation of intracellular cAMP are absolute requirements for increased NAT activity in mammalian pineal gland, we investigated whether 17β-E_2 could influence cAMP levels in pineal cells after adrenoceptor activation. Treatment of pinealocytes with ISO (1 μ*M*) increased intracellular cAMP by 100-fold compared to untreated cells, and this response was potentiated by the concurrent treatment with PHE (1 μ*M*, fig. 2A). Exposure to 17β-E_2 (1 n*M*) consistently decreased both ISO- and ISO plus PHE-induced cAMP accumulation by ~35%, without affecting basal cAMP levels (fig. 2A). The estrogen concentrations used in these experiments were equivalent to those, in vivo, present in rat blood during the estrogen surge preceding ovulation [21]. At estrogen con-

centrations up to 10 n*M* the inhibitory effect upon β-adrenoceptor-induced cAMP accumulation was dose-related; however, higher estrogen concentrations failed to elicit significant effects. In addition, estrogen exposure as short as 5 min was able to inhibit β-adrenoceptor-induced cAMP accumulation to a similar extent, provided that a latency of 48 h was allowed prior to agonist stimulation. However, when pinealocytes were exposed to the same concentration (1 n*M*) of 17β-E_2 coupled to bovine serum albumin (i.e., a derivative that does not cross the plasma membrane), no inhibitory effect upon β-adrenoceptor-induced cAMP accumulation was observed (data not shown).

PI Hydrolysis after α_1-Adrenoceptor Stimulation. To determine whether 17β-E_2 could modulate the response to adrenoceptor activation by acting, in part, on α_1-adrenoceptors, we measured the accumulation of [^{3}H]IPs in pineal cells after stimulation with PHE (1 μ*M*) in the presence of 10 m*M* LiCl. This procedure prevents [^{3}H]IP degradation by phosphatase activity, and therefore gives an accurate index of adrenoceptor-mediated PI-specific PLC [17, 18]. In addition, the assay of a total IP fraction in these conditions provides a more accurate estimation of the extent of α_1-adrenoceptor activation than does measurement of a single metabolite [22]. Treatment with PHE produced a 6-fold increase in [^{3}H]IPs formation (fig. 2B), as previously described [7], an effect that was blocked by concurrent treatment with the α_1-adrenoceptor antagonist prazosin (1 μ*M*, not shown). Exposure to 17β-E_2 (1 n*M*) reduced PHE-induced [^{3}H]IPs accumulation by ~38%, without affecting basal [^{3}H]IPs levels (fig. 2B). Short exposures of pinealocytes to the same estrogen concentration (1 n*M*, 30 s), either alone or coupled to bovine serum albumin, did not modify PHE-induced [^{3}H]IP accumulation (data not shown).

Ca^{2+} Transients after α_1-Adrenoceptor Stimulation and High K^+-Induced Depolarization. Since the potentiation effect of α_1-adrenoceptor activation on β-adrenoceptor-induced cAMP production is Ca^{2+}-dependent and mediated by the activation of Ca^{2+}/IP-dependent PKC [23], we studied the effect of 17β-E_2 exposure on the rise of $[Ca^{2+}]_i$ after α_1-adrenoceptor stimulation. As has previously been shown [24], PHE evoked a transient Ca^{2+} signal consisting on a rapid increase of $[Ca^{2+}]_i$ from 30 n*M* to 300–400 n*M*, followed by a plateau (fig. 3A), an effect that was absent when PHE was coapplied with prazosin (1 μ*M*, not shown). The presence of 17β-E_2 (1 n*M*) in the culture medium caused a ~65% reduction in $[Ca^{2+}]_i$ transients triggered by PHE, without affecting the duration of response (fig. 3B). However, a 4-fold increase in the decay

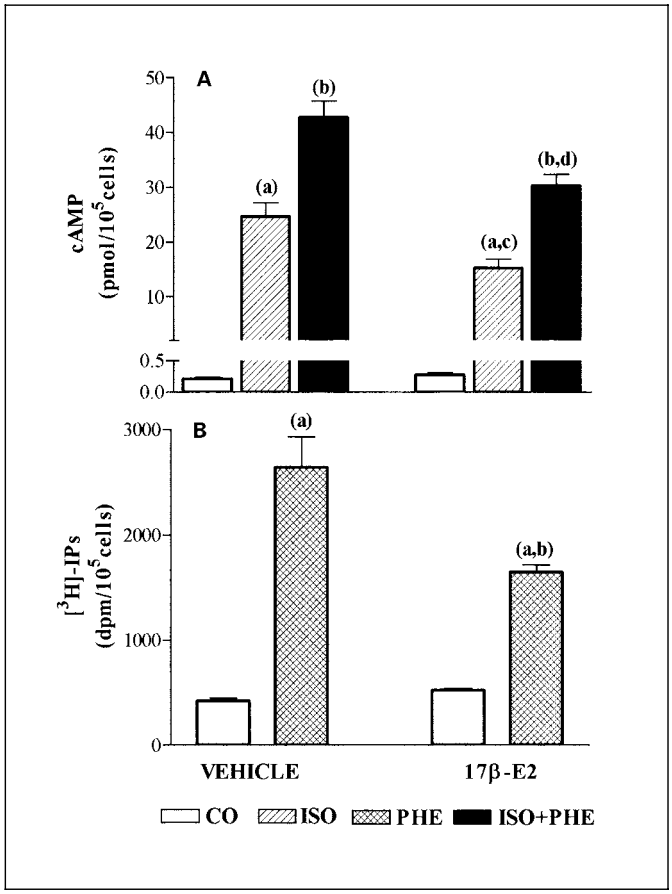

Fig. 2. Effect of 17β-E_2 on α_1/β-adrenoceptor-induced accumulation of cAMP and α_1-adrenoceptor induced PI hydrolysis in dispersed pinealocytes. **A** Pineal cells from 21-day-old ovariectomized rats were exposed to 17β-E_2 (1 n*M*, 48 h) or the vehicle (0.001% ethanol). After preincubation with a phosphodiesterase inhibitor (IBMX, 0.1 m*M*) for 60 min, they were treated with water (CO, 10 μl), ISO (1 μ*M*), or ISO plus PHE (1 μ*M* each) for 15 min. Cyclic AMP was determined in duplicate by an enzyme immunoassay kit. In this and other figures in which the experiment was repeated several times with cells from different animals, each replication was considered as one level of an additional factor in the statistical data analysis. Therefore, the p values given were those obtained accordingly. Nevertheless, each bar represents the mean ± SEM of 10–15 plates from one representative experiment. Number of experiments: 4; [a] $p < 0.001$ vs. CO; [b] $p < 0.01$ vs. ISO; [c] $p < 0.001$ vs. vehicle. **B** Pineal cells from 21-day-old ovariectomized rats were exposed to 17β-E_2 (1 n*M*, 48 h) or the vehicle (0.001% ethanol). After preincubation with *myo*-[^{3}H]inositol (3 μCi/plate) for 12 h, they were treated for 60 min with water (CO) or PHE (1 μ*M*) in the presence of 10 m*M* LiCl. [^{3}H]IP were extracted and assayed as described in the 'Materials and Methods' section. Each bar represents the mean ± SEM of 8–10 plates from one representative experiment. Number of experiments: 3; [a] $p < 0.001$ vs. CO; [b] $p < 0.001$ vs. vehicle.

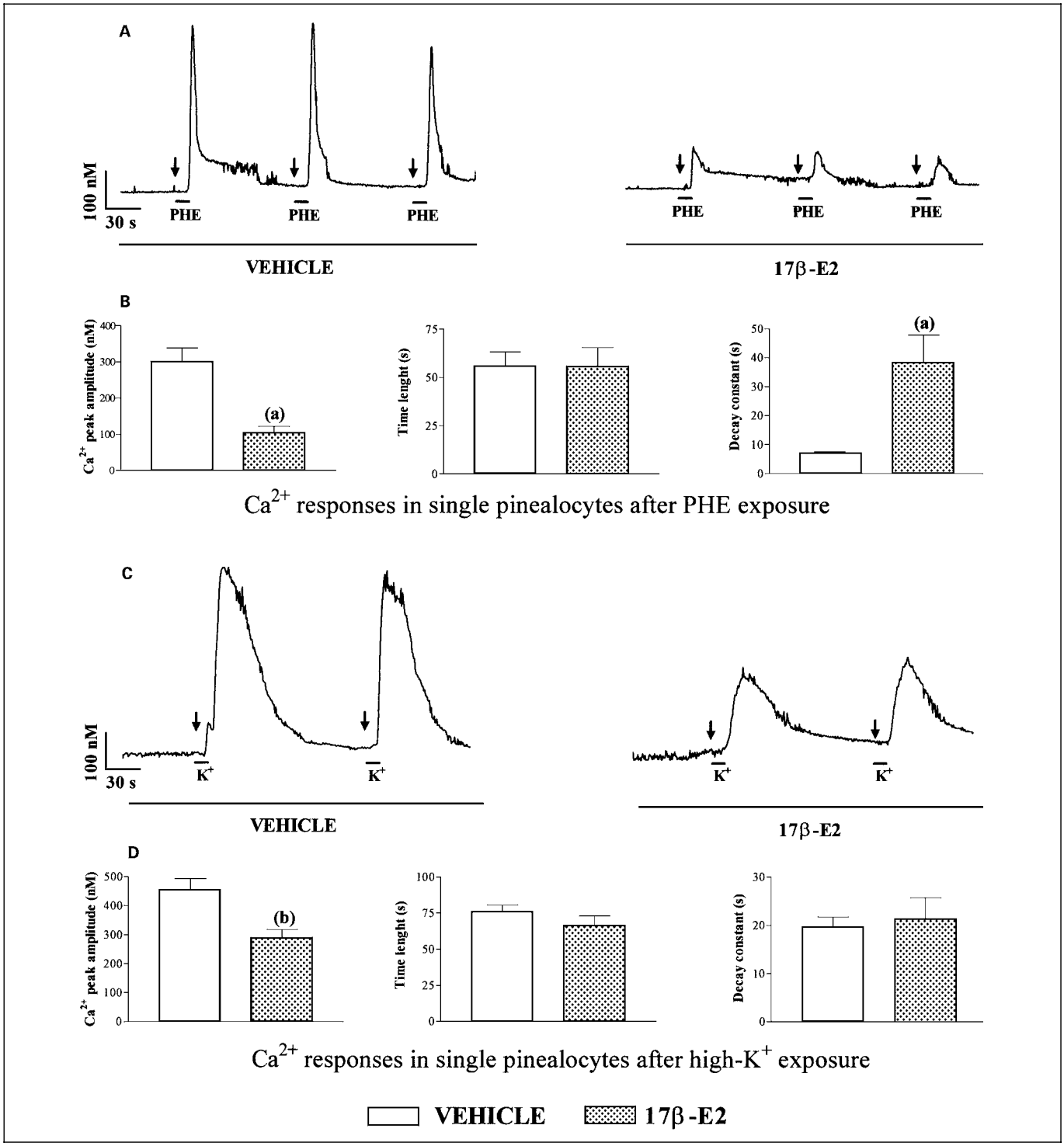

Fig. 3. Effect of estradiol on α_1-adrenoceptor-induced (**A**, **B**) and high-K^+-induced (**C**, **D**) Ca^{2+} increments in single pinealocytes. Pineal cells from 21-day-old ovariectomized rats were exposed to 17β-E_2 (1 n*M*, 48 h) or the vehicle (0.001% ethanol). Thirty minutes prior to either PHE or potassium stimulation they were loaded with Fura-2 for 15 min (see 'Ca^{2+} Measurements'). The pinealocytes were continuously perfused with control solution (in m*M*: NaCl, 140; KCl, 2.7; $MgCl_2$, 1; $CaCl_2$, 10; HEPHES, 10; glucose, 10; ascorbic acid, 5; pH 7.25–7.35), or with either test solution containing PHE (1 μ*M*) for 15 s, or with depolarizing solution containing 60 m*M* KCl for 10 s. The arrows indicate the time of PHE or KCl application. **A**, **C** Transient Ca^{2+} signals from one representative pineal cell. **B**, **D** Data representing the mean ± SEM of 30 cells (PHE stimulation) or 12 cells (KCl stimulation). [a] $p < 0.001$ vs. vehicle; [b] $p < 0.01$ vs. vehicle.

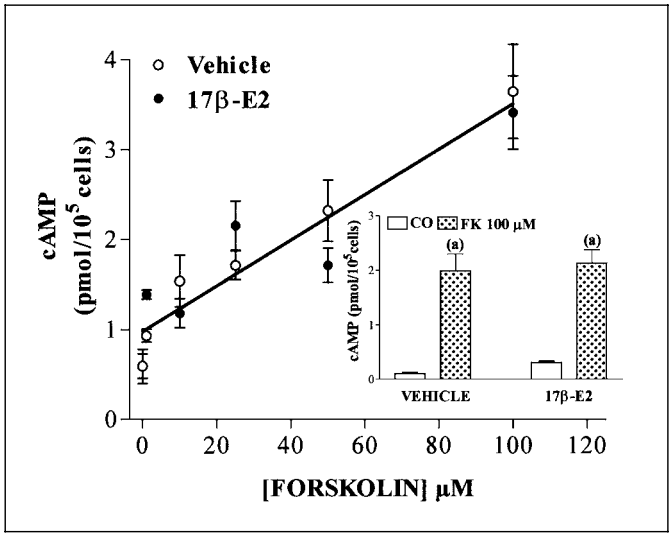

Fig. 4. Dose-dependent accumulation of cAMP in dispersed pinealocytes treated with forskolin (FK). Pineal cells from 21-day-old ovariectomized rats were exposed to 17β-E_2 (1 n*M*, 48 h) or vehicle (0.001% ethanol). Thereafter, they were incubated with FK up to 100 μ*M*, or water (10 μl) for 20 min. A dose-dependent elevation of cAMP levels was significant at $p < 0.001$, without any difference between vehicle and estradiol-treated pinealocytes. Each value represents the mean ± SEM of 8–10 plates. The effect of 100 μ*M* FK was repeated twice and the results are shown in the inset at the bottom right. [a] $p < 0.001$ vs. CO.

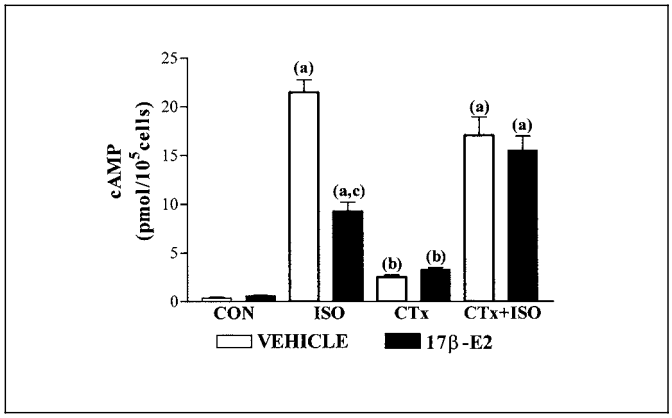

Fig. 5. Effect of 17β-E_2 on the accumulation of cAMP in dispersed pinealocytes treated with ISO or CTx. Pineal cells from 21-day-old ovariectomized rats were exposed to 17β-E_2 (1 n*M*, 48 h) or the vehicle (0.001% ethanol). After preincubation with a phosphodiesterase inhibitor (IBMX, 0.1 m*M*) for 60 min, they were treated with water (CO, 10 μl) or CTx (10 μ*M*, 30 min), followed or not by ISO (1 μ*M*, 15 min). Cyclic AMP was determined in duplicate by an enzyme immunoassay kit. Each bar represents the mean ± SEM of 10–15 plates from one representative experiment. Number of experiments: 2; [a] $p < 0.0001$ vs. CO; [b] $p < 0.01$ vs. CO; [c] $p < 0.0001$ vs. vehicle.

constant (τ) was observed following treatment with 17β-E_2. To determine whether estrogen could also modulate Ca^{2+} entry into pineal cells through voltage-gated channels, we studied the rise in $[Ca^{2+}]_i$ elicited by perfusion of pineal cells with depolarizing K^+ concentrations (60 m*M*). Figure 3C shows the increase in $[Ca^{2+}]_i$ evoked by K^+ depolarization. Previous exposure of pinealocytes to 17β-E_2 (1 n*M*) reduced by ~35% the amplitude of transient Ca^{2+} signals evoked by K^+, without altering neither the time length nor the decay constant (fig. 3D). Short exposures of pinealocytes to the same estrogen concentration (1 n*M*, 30 s) were unable to modify transient Ca^{2+} signals induced by either PHE or depolarizing K^+ solutions (data not shown).

Characterization of Estrogen-Induced Inhibition of β-Adrenoceptor Responsiveness in Female Rat Pinealocytes

Effect of 17β-E_2 on cAMP Accumulation after Treatment with Forskolin. To determine whether the attenuation of the response to ISO and ISO plus PHE and the reduction of cAMP accumulation by 17β-E_2 was a consequence of hormonal modulation exerted on AC, we measured the levels of cAMP on pinealocytes following direct stimulation with forskolin, a known activator of this enzyme. Forskolin increased pinealocyte cAMP levels in a dose-dependent manner regardless of whether or not the cells had been previously exposed to 17β-E_2 (fig. 4).

Effect of 17β-E_2 on cAMP Accumulation after Treatment with CTx. To investigate whether the inhibitory effect of estrogen could be exerted at the level of G protein activation, we measured the accumulation of cAMP in pinealocytes treated with CTx (10 μ*M*), alone or in combination with ISO (1 μ*M*). Treatment with CTx caused a 7-fold increase in pinealocytes cAMP accumulation (fig. 5). The combination of CTx and ISO treatment produced an increase in cAMP levels similar to that of ISO alone. Prior exposure to 17β-E_2 (1 n*M*) reduced ISO-dependent elevation of cAMP levels as shown before; however, it was unable to modify the accumulation of cAMP caused by either CTx alone or in combination with ISO.

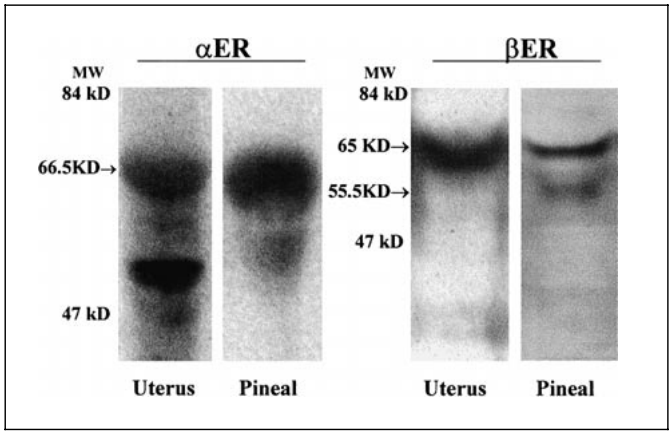

Fig. 6. Identification of ER subtypes in the uterus (positive control) and pineal cells from ovariectomized Sprague-Dawley rats by Western blot analysis. Aliquots (40 μg protein) were applied to 11% polyacrylamide gels and blotted to nitrocellulose membranes. They were developed using a specific anti-ERα monoclonal antibody (1:500 dilution, Stressgen Biotechnologies Corp., Victoria, B.C., Canada), and anti-ERβ polyclonal antibody (1:500 dilution, Upstate Biotechnology, N.Y., USA). Arrows indicate the molecular weight of ER subtypes. For further technical details, see 'Materials and Methods'.

Identification of Estrogen Receptor Subtypes in Female Rat Pineal Cells

To demonstrate the presence of nuclear estrogen receptor in female rat pineal cells, we measured ER levels in pinealocytes incubated under the same conditions as described above. Western blot analysis revealed the presence of ERα and ERβ proteins in female rat pineal cells (fig. 6). The apparent molecular weights of pineal ER proteins were identical to those observed in other estrogen-responsive rat tissues [25].

Discussion

In most species pineal melatonin synthesis and secretion are adjusted to a circadian rhythm, externally driven by the light-dark cycle [1]. Even though the importance of the internal hormonal milieu in the control of melatonin production is less clear, evidence from different species supports a modulatory role for gonadal steroids [9], including the effects of these hormones on presynaptic elements of the neural system controlling pineal activity [11, 26]. Besides differences in experimental models, the most consistent picture indicates that, in rodents, nocturnal melatonin synthesis and secretion is reduced at the time of proestrous night, apparently coinciding with the rise of estradiol preceding ovulation [11, 12]. Acute treatment of ovariectomized rats with sex steroids blocks the ISO-induced rise of pineal and serum melatonin levels [13, 27]. In addition, in peripubertal rats, ovariectomy has been shown to induce elevations of pineal melatonin levels, NAT activity, and AC activity several weeks after removal of the ovaries [10, 28], effects that were prevented by long-term treatment with estradiol benzoate. We reported previously that either ovariectomy or antiestrogen treatment of normal cycling rats increases the accumulation of cAMP in cultured pineal cells treated, in vitro, with adrenergic agonists [14]. The present study demonstrates for the first time that, in female rat pinealocytes, direct exposure to physiological concentrations of 17β-E_2 reduces the stimulation of melatonin synthesis and release evoked by simultaneous activation of both α_1- and β-adrenergic receptors. Even though the possibility that melatonin might be stored in pinealocytes and released in synchronization with an adequate signal has been postulated, simple diffusion of newly synthesized hormone is accepted as the mechanism involved in melatonin secretion [29]. Thus, the estrogen-mediated inhibition of melatonin release from pineal cells was apparently a direct consequence of a reduction in NAT activity, since a similar linear relationship between these two variables (fig. 1, inset) was observed in vehicle- and 17β-E_2-treated pinealocytes.

Second messengers responsible for the nocturnal rise of melatonin synthesis in response to accelerated noradrenergic transmission include β-adrenoceptor-induced cAMP accumulation [2] and α_1-adrenoceptor-induced PI hydrolysis and IP_3 production [6], which in turn causes the elevation of intracellular Ca^{2+} levels [7] and PKC activation [8]. Phosphorylation of CREB by cAMP-dependent protein kinase at amino acid serine 133 constitutes the main link between norepinephrine-induced activation of second messengers and the stimulation of melatonin synthesis [3–5]. Therefore, estrogen-mediated inhibition of cAMP accumulation after α_1/β-adrenoceptor activation of pineal cells, as shown here, may constitute an effective mechanism to modulate melatonin synthesis and release in response to hormonal oscillations. Accordingly, changes in estrogen concentration may act directly on pineal cells and in turn regulate several intracellular targets of noradrenergic input. As a consequence, the rhythmic secretion of melatonin into the circulation would be further modulated by oscillations in sex steroid hormone levels. Hence, the inhibition of melatonin synthesis and release observed during proestrous night [11, 12] could be related to the preovulatory rise of circulating estradiol. This may constitute part of an integrated mechanism

designed to lower circulating melatonin levels at the time of expected ovulation, in order to prevent the inhibitory effect of the indole on GnRH release [30], GnRH-induced LH secretion [31], or GnRH-dependent gene transcriptional activity in rat gonadotropes [32]. However, it must be emphasized that our static in vitro model does not reproduce the temporal course of circulating ovarian hormones during the rodent estrous cycle. Since other intracellular signals may be activated by sex steroids and modulate pineal activity in a rather complex manner, further whole animal experiments must be performed before a complete interpretation might be done.

Some clues derived from these data support the hypothesis that estrogen may exert its actions on pinealocyte β-adrenoceptor responsiveness by an ER-mediated genomic mechanism. First, while membrane actions are usually exerted at micromolar concentrations [33], these results were obtained with estrogen concentrations within the range of those occurring physiologically during the rat estrous cycle [21]. Second, estrogen effect on β-adrenoceptor sensitivity was not mimicked by 17β-E_2-bovine serum albumin (i.e., estrogen needs to enter the cells to have an effect). Third, a latency of approximately 48 h was necessary, in vitro, for the effect to be observed. It should be mentioned that, in some experiments, shorter latencies (24–36 h) seemed to be sufficient to elicit the effect; however, since 48 h appeared to give the most consistent results this time course was fixed for all experiments. Early studies using ^{3}H-17β-E_2 exchange assays had shown the presence of a nuclear receptor complex in rat pineal, the abundance of which varies with the estrous cycle or in response to estrogen treatment [34]. Also, in cultured pineal glands, 17β-E_2 induced a protein with similar electrophoretic mobility to that of estrogen-induced protein in the rat uterus [35]. More recently, the presence of ERβ mRNA in the rat pineal has been described by in situ hybridization histochemistry [36]. These results are consistent with the presence of both ERα and ERβ proteins in female rat pinealocytes, which we now report (fig. 6). Varying ratios of ERα and ERβ in estrogen-responsive cells may result in different populations of homo- and heterodimers following ligand binding [37]. Therefore, our findings could be partially explained taking into account the interactions between these two receptor types and the different and complementary transduction pathways that might be involved.

ER-mediated modification of gene expression could influence pineal β-adrenoceptor responsiveness via changes in either adrenoceptor expression or adrenoceptor binding sites [27, 38], or by affecting different proteins involved in the cAMP cascade [39–41]. Alternatively, the efficiency of signal transduction might be influenced by estrogen-dependent changes in the expression of other targets leading to β-adrenoceptor desensitization [42, 43], including cAMP-dependent protein kinase [44], PKC [45], β-adrenoceptor kinase [46], and β-arrestin [47]. As a hypothesis, ligand-dependent activation of pineal ERs might stimulate the transcription of specific proteins involved in adrenoceptor phosphorylation, thereby inducing changes in G-protein coupling and stable receptor desensitization. This explanation would agree with the present results, since 17β-E_2 reduced ISO-induced cAMP accumulation, but not that derived from either direct forskolin activation of AC or constitutive G_s protein activation by CTx. The former finding is apparently at variance with that of Hayashi and Okatani [28], who found that, in peripubertal rats, pineal AC activity was increased after long-term ovariectomy and returned to control levels by long-term estrogen treatment. However, long-term elevation of pineal AC activity in ovariectomized rats, without changes in noradrenergic input, may just reflect increased sensitivity of pineal adrenoceptors after removal of the ovaries. On the other hand, from the analysis of β-adrenergic binding parameters and the effect of nonhydrolyzable GTP analoges on competition curves in membranes from hypothalamic-preoptic area of ovariectomized estrogen-treated rats, it was suggested that estrogen may uncouple β-adrenoceptors from G_s [48]. However, slight changes in binding affinity may not permit definite explanations on membrane receptor-G protein functional interactions. Moreover, since in vivo treatment of ovariectomized rats should affect many estrogen-dependent targets, a clear relationship between estrogen action and membrane receptor function cannot be established without direct verification at cellular level. Therefore, the present results could indicate that estrogen treatment of pineal cells does not modify the levels of AC catalytic units, nor does it modulate the expression of CTx-sensitive G proteins, and that none of these proteins are the targets for estrogen modulation of β-adrenergic stimulation of cAMP accumulation in pinealocytes. On the other hand, both estrogen-dependent enhancement [49] or reduction [50] of α-adrenergic signaling pathways have been reported, and estrogen effects on the density of α_1-adrenergic binding sites have been described [37, 50–52]. Alternatively, estrogen-dependent modulation of neurotransmitter-induced PLC activity in endocrine and neural cells has been also found [53]. Since sex steroid hormone effects on signal transduction are tissue-specific, the possibility of multiple molecular targets for estrogen in the signaling pathway leading to melatonin synthesis cannot be

excluded. It should be determined whether or not this dual adrenoceptor modulation has a common origin, whether it is a consequence of ER-mediated mechanisms, and which are the protein targets that may be regulated.

In the mammalian pineal, the increase in intracellular Ca^{2+} in response to noradrenergic input takes place through α_1-adrenoceptor activation, which causes both IP_3-dependent Ca^{2+} release from intracellular stores and Ca^{2+} entry from the extracellular medium [24, 54]. In this study, exposure of pineal cells to 17β-E_2 reduced PI hydrolysis and transient Ca^{2+} signals after α_1-adrenoceptor activation. Since the effect of 17β-E_2 on PHE-induced transient Ca^{2+} consisted in a decrease in the peak amplitude of leading spikes, accompanied by an increase of their decay constant, it could be a consequence of the estrogen-dependent inhibition of α_1-adrenoceptor-induced PLC activation. However, while exposure to 17β-E_2 decreased PHE-induced [^{3}H]-IPs accumulation by ~38%, the reduction in peak amplitude of transient Ca^{2+} signals triggered by PHE was about ~65%. Therefore, it is possible that, in addition to modulating α_1-adrenoceptor sensitivity and PI turnover in pineal cells, estrogen might also be able to directly affect intracellular IP_3 receptors.

The rat pinealocytes also contain L-type Ca^{2+} channels which participate in regulating Ca^{2+} signals in response to different messengers [55], and that are activated by K^+-induced depolarization [56]. In the present experiments, 17β-E_2 also reduced transient Ca^{2+} signals induced by high K^+ solutions, although to a lesser extent than those induced by treatment with PHE. However, while 17β-E_2 decreased the peak amplitude of leading Ca^{2+} spikes induced by either α_1-adrenoceptor activation or high K^+-induced depolarization, their decay constant was significantly increased in the former case, but not affected in the latter. In other cell types, protein kinase-dependent channel phosphorylation modulates Ca^{2+} fluxes and causes significant modification in deactivation time course [57], a mechanism that might underlie the estrogen-dependent inhibition of transient Ca^{2+} signals after α_1-adrenoceptor activation. On the contrary, the inhibitory action of 17β-E_2 on depolarization-induced Ca^{2+} entry, without modifying the time course of the signal, could be due to estrogen-dependent changes in the number of membrane Ca^{2+} channels, as it has been found in anterior pituitary cells [58].

In summary, these results are consistent with estrogen-dependent changes in the amount of functional stage of several target proteins in pineal cells, which in turn would result in modulation of adrenoceptor-mediated melatonin synthesis and secretion. These effects are exerted directly on pineal cells, tentatively through hormone interactions with nuclear estrogen receptors, and display a temporal profile that is compatible with a genomic action mechanism. We are aware that the present findings may be in partial disagreement with some of the previous reports from animals treated in vivo with ovarian steroids, or from pineal explants exposed to higher estrogen concentrations [for review see ref. 9]. Also, while estrogen exposure was able to prevent the potentiating effect of PHE on ISO-induced NAT activity and melatonin production (fig. 1), it only slightly reduced the accumulation of cAMP in response to the concurrent activation of α_1- and β-adrenoceptors (fig. 2), indicating that adrenergically induced stimulation of pineal melatonin synthesis and release must be the result of several intracellular signals acting synergically. Further studies are being undertaken to answer the question of whether estrogen influences the synthesis of functional α_1- and β-adrenoceptors, the expression of other proteins that are capable of inducing adrenoceptor desensitization, or both. Analysis of ligand-dependent activation of these pathways may help to understand the modulatory role of sex steroid hormones on other neurotransmitter systems. On the other hand, estrogen-dependent changes in the pattern of melatonin secretion might be important in the human, since several clinical observations have suggested that an important relationship, albeit uncharacterized, exists between gonadal and pineal function [9]. Nocturnal circulating levels of melatonin and the amplitude of the circadian rhyhthm display profound changes during growth and sexual development, i.e., they are usually low during the first months of life, reach a peak between 1 and 3 years, and then progressively drop through adulthood [59]. Thus, developmental and age-related changes in melatonin secretion may be related, at least in part, to sex steroid hormone levels. Accordingly, estrogen exposure either by therapeutic means or from untested environmental sources [60] might affect pineal melatonin production and induce yet unknown side effects on the chronobiological system.

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References

1 Reiter RJ: Interface between the photoperiodic environment and the endocrine system. Trends Endocrine Metab 1991;2:13–29.
2 Chik CL, Ho AK: Multiple receptor regulation of cyclic nucleotides in rat pinealocytes. Prog Biophys Mol Biol 1989;53:197–203.
3 Rosenbom PH, Klein DC: Norepinephrine stimulation of pineal cyclic AMP response element-binding protein phosphorylation: Primary role of a beta-adrenergic receptor/cyclic AMP mechanism. Mol Pharmacol 1995;47: 439–449.
4 Baler R, Covington S, Klein DC: The rat arylalkylamine N-acetyltransferase gene promoter. J Biol Chem 1997;272:6979–6985.
5 Molina CA, Foulkes NS, Falli E, Sassone-Corsi P: Inducibility and negative autoregulation of CREM: An alternative promoter directs the expression of ICER, an early response repressor. Cell 1993;75:875–886.
6 Ho AK, Chik CL, Klein DC: Permissive role of calcium in α_1-adrenergic stimulation of pineal phosphatidylinositol phosphodiesterase (phospholipase C) activity. J Pineal Res 1988;5:553–564.
7 Sugden L, Sugden D, Klein DC: Essential role of calcium influx in the adrenergic regulation of cAMP and cGMP in rat pinealocytes. J Biol Chem 1986;261:11608–11612.
8 Ho AK, Thomas PT, Chik CL, Anderson WB, Klein DC: Protein kinase C: Subcellular redistribution by increased Ca^{2+} influx. J Biol Chem 1988;263:9292–9297.
9 Alonso R, Abreu P, Fajardo N: Steroid influences on pineal melatonin production; in Yu HS, Reiter RJ (eds): Melatonin: Biosynthesis, Physiological Effects and Clinical Applications. Boca Raton, CRC Press, 1993, pp 73–105.
10 Okatani Y, Kazutoshi H, Watanabe K, Morioka N, Sagara Y: Estrogen modulates the nocturnal synthesis of melatonin in peripubertal female rats. J Pineal Res 1998;24:224–229.
11 Ozaki Y, Wurtman RJ, Alonso R, Lynch HJ: Melatonin secretion decreases during the proestrous stage of the rat estrous cycle. Proc Natl Acad Sci USA 1978;75:531–534.
12 Alonso R, Abreu P, Fajardo N, Sánchez-Criado J: Progesterone does not mediate the inhibition of pineal melatonin production during the rat proestrous night. Neurosci Lett 1993;151:150–152.
13 Moujir F, Bordon R, Santana C, Abreu P, Hernandez G, Alonso R: Ovarian steroids block the isoproterenol-induced elevation of pineal melatonin production in the female rat. Neurosci Lett 1990;119:12–14.
14 Alonso R, Abreu P, Fajardo N, Hernández-Díaz F, Diaz-Cruz A, Hernández G, Sánchez-Criado J: Ovarian hormones regulate α_1- and β-adrenoceptor interactions in female rat pinealocytes. Neuroreport 1995;6:345–348.
15 Fajardo N, Abreu P, Alonso R: Determination and kinetic properties of serotonin N-acetyltransferase in bovine pineal gland by using HPLC with fluorimetic detection. J Pineal Res 1992;13:80–84.
16 Hernández G, Abreu P, Alonso R, Calzadilla CH: Determination of pineal melatonin by high-performance liquid chromatography with electrochemical detection: Application for rhythm studies and tissue explants. J Pineal Res 1990;8:11–19.
17 Sandmann J, Wurtman RJ: Stimulation of phospholiphase D activity in human neuroblastoma (LA-N-2) cells by activation of muscarinic acetylcholine receptors or by phorbol esters: Relationship to phosphoinositide turnover. J Neurochem 1991;56:1312–1319.
18 Berridge MJ, Downes CP, Hanley MR: Lithium amplifies agonist-dependent phophatidylinositol responses in brain and salivary glands. Biochem J 1982;206:587–595.
19 Grynkiewicz C, Poenie M, Tsien RY: A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. J Biol Chem 1985;260:3440–3450.
20 Snedecor GW, Cochran WG: 1980 Statistical Methods. Ames, Iowa State University Press, 1985.
21 Smith MS, Freeman ME, Neill JD: The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: Prolactin, and gonadotrophin and steroid levels associated with rescue of the corpus luteum. Endocrinology 1975;96:219–226.
22 Godfrey PP: Inositol lipids and phosphates; in Milligan G (ed): Signal Transduction: A Practical Approach. Oxford, IRL Press, 1992, pp 105–121.
23 Sugden D, Vanecek J, Klein DC, Thomas TP, Anderson WB: Activation of protein kinase C potentiates isoprenaline-induced cyclic AMP accumulation in rat pinealocytes. Nature 1985; 314:359–361.
24 Marin A, Ureña J, Tabares L: Intracellular calcium release mediated by noradrenaline and acetylcholine in mammalian pineal cells. J Pineal Res 1996;21:15–28.
25 Telleria CM, Zhong L, Deb S, Srivastava RK, Park KS, Sugino N, Park-Sarge OK, Gibori G: Differential expression of the estrogen receptors alpha and beta in the rat corpus luteum of pregnancy: Regulation by prolactin and placental lactogens. Endocrinology 1998;139:2432–2442.
26 Cardinali DP, Nagle CA, Gópez E, Rosner JM: Norepinephrine turnover in the rat pineal gland: Acceleration by estradiol and testosterone. Life Sci 1975;16:1717–1724.
27 Yie SM, Brown GM: Effects of sex hormones on the pineal response to isoproterenol and on pineal beta adrenergic receptors. Neuroendocrinology 1995;62:93–100.
28 Hayashi K, Okatani Y: Mechanisms underlying the effects of estrogen on nocturnal melatonin synthesis in peripubertal female rats: Relation to norepinephrine and adenylate cyclase. J Pineal Res 1990;26:178–183.
29 Pang SF, Yip PCY: Secretory patterns of pineal melatonin in the rat. J Pineal Res 1988;5:279–292.
30 Akema T, Chiba A, Ikeda T, Nagami Y, Kimura F, Toyoda J: Melatonin inhibits naloxone-induced luteinizing hormone release in ovariectomized estrogen-primed rats. J Neuroendocrinol 1997;9:849–857.
31 Vanecek J: Melatonin inhibits release of luteinizing hormone (LH) via decrease of $[Ca^{2+}]i$ and cyclic AMP. Physiol Res 1998;47:329–335.
32 Sumova A, Vanecek J: Melatonin inhibits GnRH-induced increase of cFOS immunoreactivity in neonatal rat pituitary. J Neuroendocrinol 1997;9:135–139.
33 Valverde MA, Rojas P, Amigo J, Cosmelli, Orio P, Bahamonde MI, Mann GE, Vergara C, Latorre R: Acute activation of Maxi-K channels *(hSlo)* by estradiol binding to the β subunit. Science 1999;285:1929–1931.
34 Cardinali DP: Nuclear receptor estrogen complex in the pineal gland: Modulation by sympathetic nerves. Neuroendocrinology 1977;24: 333–346.
35 Mizobe F, Kurokawa M: Induction of a specific protein by oestradiol in rat pineals in culture. FEBS Lett 1978;87:45–48.
36 Shughrue PJ, Lane MV, Merchenthaler I: Comparative distribution of estrogen receptor-α and -β mRNA in the rat central nervous system. J Comp Neurol 1997;388:507–525.
37 Kupier GG, Gustafsson JA: The novel estrogen receptor-β subtyPHE: Potential role in the cell- and promoter-specific actions of estrogens and anti-estrogens. FEBS Lett 1997;40:87–90.
38 Karkanias GB, Ansonoff MA, Etgen AM: Estradiol regulation of α_{1b}-adrenoceptor mRNA in female rat hypothalamus-preoptic area. J Neuroendocrinol 1996;8:449–455.
39 Ostrowska A: The activation of protein kinase A by cyclic AMP is influenced by oestradiol and progesterone in supernatants from the anterior pituitary but not from hypothalamus of the female rat. Biol Cell 1994;81:223–226.
40 Cohen-Tannoudji J, Mhaouty S, Elwardy-Merezak J, Lecrivain J-L, Robin M-T, Legrand C, Maltier J-P: Regulation of myometrial Gi2, and Gq expression during pregnancy: Effects of progesterone and estradiol. Biol Reprod 1995; 53:55–64.
41 Monroe JJ, Tashjian H Jr: Pretreatment with 17β-estradiol attenuates basal- and PTH-stimulated membrane adenylyl cyclase activity in human osteoblast-like SAOS-2 cells. Biochem Biophys Res Commun 1996;225:320–325.
42 Freedman NJ, Lefkowitz RJ: Desensitization of G protein-coupled receptors. Rec Prog Horm Res 1996;51:319–353.
43 Daaka Y, Luttrell LM, Lefkowitz RJ: Switching of the coupling of the β_2-adrenergic receptor to different G proteins by protein kinase A. Nature 1997;390:88–91.
44 Benovic JL, Pike LJ, Cerione RA, Staniszewski C, Yoshimasa T, Codina J, Caron MG, Lefkowitz RJ: Phosphorylation of the mammalian β-adrenergic receptor by cyclic AMP-dependent protein kinase. J Biol Chem 1985;260:7094–7101.

45 Bouvier M, Leeb-Lundberg LM, Benovic JL, Caron MG, Lefkowitz RJ: Regulation of adrenergic receptor function by phosphorylation. J Biol Chem 1987;262:3106–3113.
46 Benovic JL, Strasser RH, Caron MG, Lefkowitz RJ: α-Adrenergic receptor kinase: Identification of a protein kinase that phosphorylates the agonist-occupied form of the receptor. Proc Natl Acad Sci USA 1986;83:2797–2801.
47 Lohse MJ, Benovic JL, Codina J, Caron MG, Lefkowitz RJ: β-Arrestin: A protein that regulates β-adrenergic receptor function. Science 1990;248:1547–1550.
48 Ungar S, Makmann MH, Morris SA, Etgen AM: Estrogen uncouples β-adrenergic receptor from the stimulatory guanine nucleotide-binding protein in female rat hypothalamus. Endocrinology 1993;133:2818–2826.
49 Petitti N, Etgen AM: α_1-Adrenoreceptor augmentation of β-stimulated cAMP formation is enhanced by estrogen and reduced by progesterone in rat hypothalamic slices. J Neurosci 1990;10:2842–2849.
50 Karkanias GB, Etgen AM: Estradiol attenuates α_2-adrenoreceptor-mediated inhibition of hypothalamic norepinephrine release. J Neurosci 1993;13:3448–3455.
51 Etgen AM, Karkanias GB: Estradiol regulates the number of α_1 but not β or α_2 noradrenergic receptors in hypothalamus of female rats. Neurochem Int 1990;16:1–9.
52 Weiland NG, Wise PM: Estrogen alters diurnal rhythm of alpha 1-adrenergic receptor densities in selected brain regions. Endocrinology 1987; 121:1751–1758.
53 Schoepp DD, Bailly DA: Estrogen modulation of angiotensin-stimulated phosphoinositide hydrolysis in slices of the anterior pituitary. Neurochem Int 1987;11:149–154.
54 Saez JC, Moreno AP, Spray DC: Norepinephrine induces Ca^{2+} release from intracellular stores in rat pinealocytes. J Pineal Res 1994;16: 57–64.
55 Chik CL, Li B, Karpinski E, Ho A: Regulation of L-type Ca^{2+} channel currents in rat pinealocytes: Role of basal phosphorylation. J Neurochem 1999;72:73–80.
56 Letz B, Schomerus C, Maronde E, Korf H-W, Korbmacher C: Stimulation of a nicotinic Ach receptor causes depolarization and activation of L-type Ca^{2+} channels in rat pinealocytes. J Physiol 1997;499:329–340.
57 Parekh AB, Terlau H: Effects of protein phosphorylation on the regulation of capacitative calcium influx in Xenopus oocytes. Pflüger's Arch Eur J Physiol 1996;432:14–25.
58 Ritchie AK: Estrogen increases low voltage-activated calcium current density in GH_3 anterior pituitary cells. Endocrinology 1993;132: 1621–1629.
59 Waldhauser F, Kovacs J, Reiter E: Age-related changes in melatonin levels in humans and its potential consequences for sleep disorders. Exp Gerontol 1998;33:759–772.
60 Sharpe RM, Fisher JS, Millar MM, Jobling S, Sumpter JP: Gestational and lactational exposure of rats to xenoestrogens results in reduced testicular size and sperm production. Environ Health Perspect 1995;12:1137–1143.