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# Estrogens Cross-Talk to $\alpha_{1b}$-Adrenergic Receptors[S]

Aliesha González-Arenas, Beatriz Aguilar-Maldonado, S. Eréndira Avendaño-Vázquez, and J. Adolfo García-Sáinz

*Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Ciudad de México, México*

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## ABSTRACT

$\beta$-Estradiol induced $\alpha_{1b}$-adrenergic receptor desensitization in U373 MG cells stably expressing $\alpha_{1b}$-adrenoceptors, as evidenced by a reduction in the adrenergic-mediated $Ca^{2+}$ mobilization; desensitization was associated with receptor phosphorylation and internalization. These effects of $\beta$-estradiol were rapid (taking place during 15 min) and were blocked by the estrogen receptor antagonist ICI 182,780 (faslodex). Likewise, inhibitors of phosphoinositide 3-kinase [wortmannin and 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002)] and of protein kinase C [staurosporine, 3-[1-[3-(amidinothio)propyl-1*H*-indol-3-yl]-3-(1-methyl-1*H*-indol-3-yl) maleimide (Ro31-8220), and rottlerin] blocked the desensitization and phosphorylation of $\alpha_{1b}$-adrenoceptors induced by estradiol. The formation of a complex was suggested by coimmunoprecipitation assays. The regulatory and catalytic subunits of phosphoinositide 3-kinase (p85 and p110) and protein kinase C $\delta$ were associated with $\alpha_{1b}$-adrenoceptors in the absence of stimulus, and such association further increased in a dynamic fashion in response to $\beta$-estradiol. In cells cotransfected with the estrogen receptor $\alpha$ and $\alpha_{1b}$-adrenoceptors, $\beta$-estradiol induced phosphorylation, desensitization and internalization of the adrenergic receptors; pretreatment with ICI 182,780 inhibited these effects. Our data support the idea that estrogens modulate $\alpha_{1b}$-adrenergic action through estrogen receptor $\alpha$.

$\alpha_1$-Adrenoceptors ($\alpha_1$-ARs) mediate many of the actions of the natural catecholamines adrenaline and noradrenaline in the body's cells, including those in the cardiovascular, genitourinary and central nervous systems (García-Sáinz et al., 1999). Consequently, knowledge of the molecular events that control their function is of major importance for the understanding of the marvelous physiological balance that we call "health". These receptors also participate in the pathogenesis of diseases, such as hypertension or benign prostatic hypertrophy, and synthetic agonists and antagonists are currently used in everyday medical practice.

$\alpha_{1b}$-ARs are members of the superfamily of the seven transmembrane domain G-protein-coupled receptors. The function of these receptors is regulated through many different processes with different time frames. Receptor phosphorylation seems to be a cardinal initial event in desensitization of G protein-coupled receptor signaling. Two major types of desensitization exist: homologous and heterologous desensitization. Homologous desensitization, in which receptors occupied by agonists reduce their responsiveness, mainly involves receptor phosphorylation by G protein-coupled receptor kinases. Heterologous desensitization, in which activation of nonadrenergic receptors desensitizes adrenergic receptors, mainly involves phosphorylation of receptors and other signaling entities by second messenger-activated kinases, such as protein kinases A and C (García-Sáinz et al., 2000; Ferguson, 2001).

Receptor phosphorylation increases the affinity of receptors for a family of cytoplasmic proteins known as arrestins. Recruitment of arrestins desensitizes signaling by blocking G-protein interaction with receptors. In addition, arrestins act as adapters to facilitate the endocytosis of G protein-coupled receptors mediated by clathrin-coated pits (Pitcher et al., 1998; Penn et al., 2000). Internalized receptors are ultimately dephosphorylated and recycled back to the plasma membrane or ubiquitin-targeted for degradation (Pitcher et al., 1995, 1998).

In organisms, cells are in contact with the internal milieu, the composition of which is constantly changing to preserve homeostasis. Under the action of hormones, neurotransmitters, autocoids, and growth factors, cells modify their responsiveness to many stimuli. During the last few years, our group has studied how the activation of a variety of nonad-

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**ABBREVIATIONS:** AR, adrenergic receptor; PI3K, phosphoinositide 3-kinase; ER, estrogen receptor; LY294002, 2-(4-morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one; Ro31-8220, 3-[1-[3-(amidinothio)propyl-1*H*-indol-3-yl]-3-(1-methyl-1*H*-indol-3-yl) maleimide; ICI 182,780, faslodex; HEK, human embryonic kidney; EGFP, enhanced green fluorescent protein; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

renergic receptors can induce $\alpha_{1b}$-AR phosphorylation. Thus, it has been observed that activation of seven transmembrane domain receptors coupled to $G_{q/11}$ (such as endothelin $ET_A$ receptors or bradykinin B2 receptors) (Vázquez-Prado et al., 1997; Medina et al., 1998) or $G_i$ (such as lysophosphatidic acid receptors) (Casas-González et al., 2003) or receptors with intrinsic tyrosine kinase activity (such as those for epidermal growth factor, platelet-derived growth factor, and insulin) (Medina et al., 2000; García-Sáinz et al., 2004) can induce $\alpha_{1b}$-AR phosphorylation and desensitization. Protein kinase C and phosphoinositide 3-kinase (PI3K) are key participants in these effects (reviewed by García-Sáinz et al., 2000).

Estrogen receptors are steroid hormone nuclear receptors which, when bound to estrogens, modulate the transcriptional activity of target genes. There are two estrogen receptor isoforms that arise from different genes: ER-$\alpha$ and ER-$\beta$ (Koike et al., 1987; Kuiper et al., 1996). Despite the clarity with which the estrogen receptors have been shown to act as transcriptional factors, many studies support the notion that not all biological effects of estrogens, such as 17$\beta$-estradiol, are accomplished via regulation of gene expression (Simoncini et al., 2000; Segars and Driggers, 2002). Estrogens induce a rapid activation of diverse signal transduction pathways in a period (seconds to minutes) that is inconsistent with the possibility of being mediated by synthesis of RNA or protein; furthermore, these effects are usually insensitive to inhibitors of RNA and protein synthesis (Lagrange et al., 1997).

It was recently observed that ER-$\alpha$ directly associates with the p85$\alpha$ regulatory subunit of PI3K in a hormone-dependent manner leading to activation of the PI3K/Akt signaling pathway (Lagrange et al., 1997; Simoncini et al., 2000; Segars and Driggers, 2002). Because our previous work suggested a key role for PI3K in $\alpha_{1b}$-AR phosphorylation and desensitization, we tested whether 17$\beta$-estradiol had any effect. Our data showed that activation of ER-$\alpha$ induced $\alpha_{1b}$-AR phosphorylation, desensitization and internalization; these effects seem to involve the interaction of signaling entities such as receptors and kinases forming a signaling complex (signalplex) (Neve, 2005).

## Materials and Methods

**Materials.** Dulbecco's modified Eagle's medium, fetal bovine serum, and other reagents used for cell culture were obtained from Invitrogen. Noradrenaline, phentolamine, 17$\beta$-estradiol, wortmannin, LY294002, staurosporine, Ro31-8220, rottlerin, and protease inhibitors were from Sigma Chemical Co (St. Louis, MO). ICI 182,780 was from Tocris Cookson Inc. (Ellisville, MO). Sepharose-coupled protein A was from Upstate Biotechnology (Lake Placid, NY). Nitrocellulose membranes were from Bio-Rad (Hercules, CA). All primary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), except the anti-$\alpha_{1b}$-AR, which was generated in our laboratory against carboxyl terminus decapeptide of the hamster $\alpha_{1b}$-AR (Vázquez-Prado et al., 1997). This antiserum immunoprecipitates $\alpha_{1b}$-ARs with high efficacy (~80% of photolabeled receptors) and selectivity (Vázquez-Prado et al., 1997). Secondary antibodies were from Zymed Laboratories (South San Francisco, CA) and the chemiluminescence kits were obtained from Pierce (Rockford, IL). [$^{32}$P]$P_i$ (8500–9120 Ci/mmol), [aryl-$^{125}$I]azidoprazosin (2200 Ci/mmol), and [$^3$H]prazosin (78 Ci/mmol) were from PerkinElmer Life and Analytical Sciences (Boston, MA).

**Cell Lines and Culture.** HEK-293 transfected cells with human $\alpha_{1b}$-AR-EGFP without or with human ER-$\alpha$ and U373 MG wild type or transfected with the hamster $\alpha_{1b}$-AR were cultured in glutamine-containing high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 1% pyruvate, 1% nonessential amino acids, 300 $\mu$g/ml neomycin analog G-418 sulfate, 100 $\mu$g/ml streptomycin, 100 U/ml penicillin, and 0.25 $\mu$g/ml amphotericin B at 37°C under a 95% air/5% $CO_2$ atmosphere. In all experiments, U373 MG wild type or transfected confluent cells were serum-deprived for 48 h in unsupplemented Dulbecco's modified Eagle's medium (without phenol red), and HEK-293 transfected cells were serum-deprived for 24 h under the same conditions.

**Receptor Binding and Photoaffinity Labeling.** Membranes were prepared and radioligand binding and photoaffinity labeling studies were performed as described previously (Vázquez-Prado et al., 1997) (see Supplemental Data 1).

**Intracellular Free Calcium Concentration ($[Ca^{2+}]_I$) Measurements.** Determinations were made in cells loaded with Fura-2 acetoxymethyl ester using an Aminco-Bowman Series 2 Spectrometer and $[Ca^{2+}]_i$ was calculated as described previously (Grynkiewicz et al., 1985) (see Supplemental Data 1).

**Determination of $\alpha_{1b}$-AR Phosphorylation.** Receptor phosphorylation studies were performed as described previously (Vázquez-Prado et al., 1997) with some modifications (see Supplemental Data 1).

**Western Blot and Akt Phosphorylation Assays.** After treatment with or without ICI 182,780, inhibitors, and/or 17$\beta$-estradiol, cells were washed with ice-cold phosphate-buffered saline and lysed for 1 h on ice as described previously (see Supplemental Data 1). Cell lysates were centrifuged at 12,700*g* for 15 min, and proteins in supernatants were quantified. In all immunoblot assays, equal amounts of protein (70 $\mu$g) were separated by electrophoresis on 10% SDS-polyacrylamide gel electrophoresis. Proteins were electrotransferred to nitrocellulose membranes, and immunoblotting was performed. Incubation with ER-$\alpha$, Akt, or p-Akt selective antibody was for 12 h at 4°C and with the secondary antibody for 30 min at room temperature. Super signal enhanced chemiluminescence kits were used, exposing the membranes to X-Omat X-ray film.

**Coimmunoprecipitation Studies.** $\alpha_{1b}$-ARs were immunoprecipitated as described previously (Vázquez-Prado et al., 1997) with minor modifications. In brief, after treatment with ICI 182,780, inhibitors, and/or 17$\beta$-estradiol, cells were washed with ice-cold phosphate-buffered saline and lysed for 1 h as described above. Cell lysates were centrifuged at 12,700*g* for 15 min, and the supernatants were incubated overnight at 4°C with the anti-$\alpha_{1b}$-AR or ER-$\alpha$ antiserum (Vázquez-Prado et al., 1997) and protein A-Sepharose. After two washes with 50 mM HEPES, 50 mM $NaH_2PO_4$, 100 mM NaCl, pH 7.4, and 0.1% Nonidet P-40, the immune complexes were denatured by boiling in SDS sample buffer containing 5% $\beta$-mercaptoethanol and subjected to 7.5% SDS-polyacrylamide gel electrophoresis. Proteins were electrotransferred to nitrocellulose membranes and immunoblotting was performed. Incubation with the $\alpha_{1b}$-AR, ER-$\alpha$, PI3K 110$\alpha$, PI3K 85, or protein kinase C isoenzyme-selective antibodies was for 12 h at 4°C and with the secondary antibody for 30 min at room temperature. Super signal-enhanced chemiluminescence kits were used, exposing the membranes to X-Omat X-ray films; all coimmunoprecipitations were assessed by densitometric analysis.

**Human $\alpha_{1b}$-AR Constructs and Expression in U373 MG and HEK-293 Cells.** The full-length cDNA encoding the human $\alpha_{1b}$-AR [generously provided by Dr. Gozoh Tsujimoto (Department of Genomic Drug Discovery Science, Faculty of Pharmaceutical Sciences, Kyoto University, Kyoto, Japan)] was amplified and its stop codon was removed by polymerase chain reaction before insertion into BglII/ EcoRI sites of pEGFP-N1 vector (Clontech, Mountain View, CA) to generate a receptor tagged at the C terminus with the enhanced green fluorescent protein (EGFP). Primers used were: $\alpha_{1B}$BglII, 5′GGAAGATCTCCACCATGAATCCCGACCT-

GGACACCG3′; and $\alpha_{1B}$EcoRI, 5′CCGGAATTCCAAACTGCCCGGGCGCCAG3′. The presence of the correct nucleotide sequences was verified by double-stranded DNA sequencing.

HEK-293 and U373 MG cells were transfected with the $\alpha_{1b}$-AR-EGFP plasmid construction using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. As a control, U373 MG cells were transfected with the pEGFP-N1 vector. Assays were made 48 h after transfection.

**Human ER-$\alpha$ Transfection in HEK-293 Cells.** Cells were co-transfected with plasmids containing the human $\alpha_{1b}$-AR-EGFP and human ER-$\alpha$ using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. ER-$\alpha$ cDNA was inserted in pT7B vector [generously provided by Dr. Fernando Larrea and Rocío García (Instituto Nacional de Ciencias Médicas y Nutrición "Salvador Zubirán", Ciudad de México, México)].

**Confocal Microscopy.** Confocal images were obtained using a MCR 1024 Bio-Rad confocal system interfaced to a TMD 300 Nikon Diaphot inverted light microscope with a 100×/1.3 numeric aperture glycerol-immersion objective. EGFP was excited using the 488-nm line of a krypton/argon laser and the emitted fluorescence detected with a band pass filter at 515 to 540 nm. All images were obtained using a numeric aperture of 1.3 and the same laser percentage, iris aperture, and gain. Operating the laser at a low power setting (97–99% attenuation) substantially reduced photobleaching and photodamage. Confocal images were viewed, processed, and converted to TIFF format with Todd Clark's program Confocal Assistant 4.2. Assays were made with cells growing on a coverglass-based chamber. When stimuli were added, the solution was pipetted directly into the chamber.

**Statistical Analysis.** All data were analyzed by using a one-way analysis of variance followed by Bonferroni's post test. A value of $p < 0.05$ was considered statistically significant and it is so stated in the figure legends. Prism (ver. 4; GraphPad Software, San Diego, CA) was used for calculating probability values.

## Results

**ER$\alpha$ and $\alpha_{1b}$-AR Expression in U373 MG Cells.** U373 MG is an astrocytoma cell line that constitutively expresses ER-$\alpha$ (Fig. 1A). The expression of ER-$\beta$ is either very low or absent because it was not clearly detected by Western blot analysis; T47D cells that express both receptors (ER-$\alpha$/ER-$\beta$ ratio approximately 9:1) (Strom et al., 2004) were used for comparison (Fig. 1A). It has been reported that U373 MG cells express a low density of $\alpha_{1b}$-ARs that are coupled to phosphoinositide hydrolysis and calcium mobilization (Arias-Montaño et al., 1999). However, we observed that under our culture conditions, only a few cells weakly responded to $\alpha_{1b}$-AR stimulation. In the experiments with these cells, we were essentially unable to detect a functional response or receptor expression by photoaffinity labeling (Fig. 1C) or radioligand binding studies (data not shown). Therefore, we transfected these cells with the hamster $\alpha_{1b}$-AR, and a stable cell line (U373 $\alpha_{1b}$) was obtained that was used in all subsequent experiments. Radioligand binding, photoaffinity labeling, and Western blotting evidenced the stable expression of these adrenoceptors. [$^3$H]Prazosin binding saturation isotherms showed a saturable number of sites ($B_{max}$ 229 ± 13.87 fmol/mg of protein), and the Rosenthal (Scatchard) transformation of the data showed a straight line consistent with a homogenous population of $\alpha_{1b}$-ARs with high affinity for the radioligand ($K_D$, 0.36 ± 0.07 nM; means ± S.E.M., $n$ = 4). These levels of expression are within what has been observed in cells that endogenously express these receptors. Photoaffinity labeling using [aryl-$^{125}$I]azidoprazosin allowed the detection of a major band of ≈80 to 85 kDa, corresponding to the receptor, and a minor one of ≈ 40 kDa, probably a proteolytic product (Fig. 1C); the labeling of these bands was specific as evidenced by competition by the $\alpha_1$-AR antagonist, phentolamine (Fig. 1C). Data using membranes from rat-1 fibroblasts are presented for comparison (Fig. 1C) (Vázquez-Prado et al., 1997).

**Effect of 17$\beta$-Estradiol on $\alpha_{1b}$-AR Function and Roles of PI3K and Protein Kinase C.** The functional effect of $\alpha_{1b}$-ARs was next examined. It was observed that noradrenaline (NA) increased the intracellular concentration of calcium ($[Ca^{2+}]_i$) ≈ 2-fold almost immediately after addition to the cells (Fig. 2A). The effect of noradrenaline was concentration-dependent with an $EC_{50}$ value of 0.58 $\mu$M (Fig. 2B). It is noteworthy that cell treatment with 17$\beta$-estradiol for 15

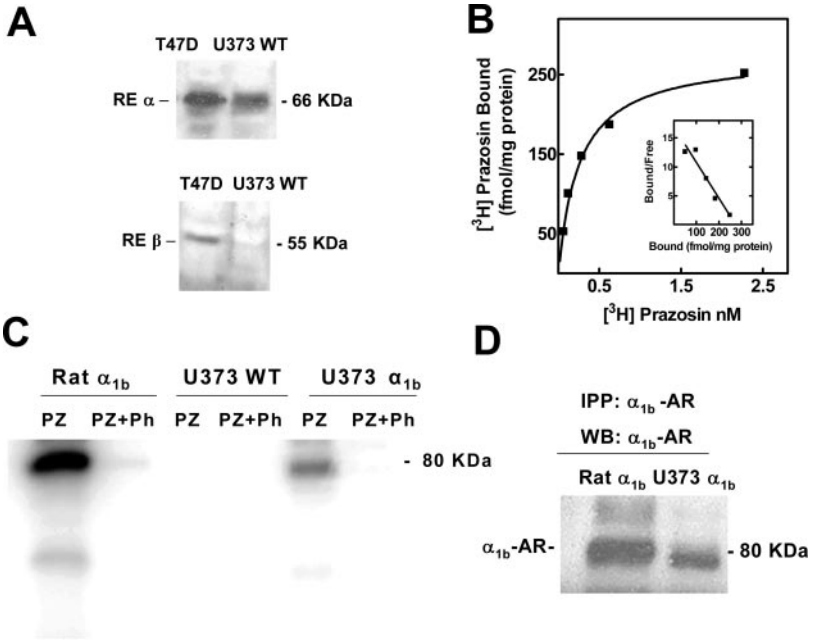


**Fig. 1.** Endogenous ER-$\alpha$ and $\alpha_{1b}$-AR transfection in U373 MG wild-type cells. A, T47D and U373 MG (U373 WT) cells were lysed, proteins (70 $\mu$g) were separated by electrophoresis, and the gels were electrotransferred for Western blot detection of ER-$\alpha$ and ER-$\beta$. B, representative binding saturation isotherm and the Rosenthal transformation of the data using membranes from U373 MG stably expressing $\alpha_{1b}$-ARs (U373 $\alpha_{1b}$). C, photolabeling of $\alpha_{1b}$-ARs with [aryl-$^{125}$I]azidoprazosin (PZ) in the absence or presence of phentolamine (Ph) in U373 WT cells, rat fibroblasts stably expressing $\alpha_{1b}$-ARs (Rat$\alpha_{1b}$) and U373$\alpha_{1b}$ cells. D, $\alpha_{1b}$-ARs were immunoprecipitated from lysed Rat-1 cells expressing $\alpha_{1b}$-ARs and U373 $\alpha_{1b}$ cells and subjected to electrophoresis, electrotransference, and Western blot analysis. In all cases, the data are representative of three or four experiments with similar results.

min decreased in a concentration-dependent fashion the increase in $[Ca^{2+}]_i$ induced by a maximally effective concentration of noradrenaline (100 $\mu$M) (Fig. 2, A and C). The changes in $[Ca^{2+}]_i$ were not altered by yohimbine or propranolol, $\alpha_2$- and $\beta$-adrenergic antagonists, respectively, but were completely blocked by phentolamine (Supplemental Data 2). Treatment with 17$\beta$-estradiol diminished (~50–60%) the increase in $[Ca^{2+}]_i$ induced by noradrenaline (Fig. 2C) with an $EC_{50}$ value of 150 nM. The effect of 17$\beta$-estradiol treatment (15 min) was abolished if cells were preincubated for 10 min with 2 $\mu$M ICI 182,780, a selective estrogen receptor antagonist (Fig. 3A). ICI 182,780 did not block the small effect of 17$\beta$-estradiol and did not by itself induce any detectable change in basal $[Ca^{2+}]_i$ or alter the effect of noradrenaline (Fig. 3A).

Protein kinase C and PI3K are key enzymes in $\alpha_{1b}$-AR heterologous desensitization and phosphorylation (Vázquez-Prado et al., 1997; Medina et al., 1998, 2000; García-Sáinz et al., 2004). The effect of different protein kinase C and PI3K inhibitors was tested to determine whether these enzymes play a role in the $\alpha_{1b}$-AR desensitization induced by 17$\beta$-estradiol. The PI3K inhibitors wortmannin (100 nM) and LY294002 (1 $\mu$M) blocked the effect of 17$\beta$-estradiol on $\alpha_{1b}$-AR-mediated increase in $[Ca^2]_i$; similarly, the protein kinase C inhibitors staurosporine, Ro31-8220, and rottlerin abolished the effect of 17$\beta$-estradiol on the $\alpha_{1b}$-adrenergic action (Fig. 3B). None of these inhibitors had any effect on the increase in $[Ca^{2+}]_i$ induced by noradrenaline (Supplemental Data 3).

**17$\beta$-Estradiol Induces $\alpha_{1b}$-AR Phosphorylation; PI3K and Protein Kinase C Participate in This Event.** The possibility that the desensitization of $\alpha_{1b}$-ARs induced by 17$\beta$-estradiol could be related to receptor phosphorylation was examined next. TPA and noradrenaline were used as positive controls of $\alpha_{1b}$-ARs phosphorylation (Fig. 4A). As shown in Fig. 4, 1 $\mu$M 17$\beta$-estradiol induced phosphorylation of $\alpha_{1b}$-adrenergic receptors (~60% increase over basal phosphorylation) and ICI 182,780 (estrogen receptor antagonist) blocked this effect. Inhibitors were used to determine whether protein kinase C and PI3K participated in the $\alpha_{1b}$-ARs phosphorylation induced by 17$\beta$-estradiol. Wortmannin and LY294002 (inhibitors of PI3K) and staurosporine, Ro31-8220, and rottlerin (inhibitors of protein kinase C) abolished this effect (Fig. 4B). None of these inhibitors had any effect on basal receptor phosphorylation (data not shown).

**PI3K Activity Increases in Response to 17$\beta$-Estradiol.** Akt is one of the most important substrates of PI3K in vivo. Therefore, the action of 17$\beta$-estradiol on the phosphorylation state of Akt was tested. Immunoblot analysis demonstrated that 17$\beta$-estradiol treatment led to significant increase in p-Akt (2-fold over basal phosphorylation state) with no change in total Akt levels (Fig. 5). It was also observed that this increase in phosphorylation of Akt was mediated by estrogen receptors (i.e., the effect was blocked by ICI 182,780) and involved PI3K (i.e., the effect was blocked by LY294002 and wortmannin) (Fig. 5). Wortmannin consistently decreased both basal and 17$\beta$-estradiol-stimulated p-Akt/Akt ratio below the nonstimulated values (Fig. 5), whereas the other inhibitors did not affect basal ratio (data not shown).

**PI3K and Protein Kinase C $\delta$ Associate with $\alpha_{1b}$-ARs in a Dynamic Fashion.** To get further insight on the mo-

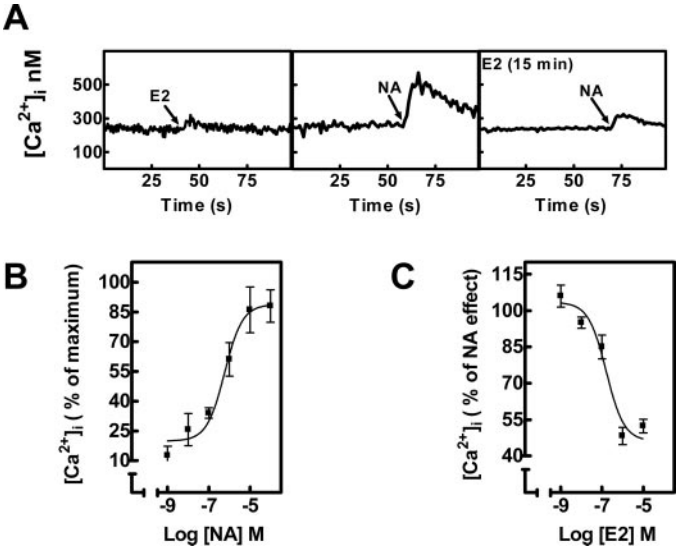


**Fig. 2.** Effect of different concentrations of noradrenaline (NA) and 17$\beta$-estradiol ($E_2$) on intracellular free calcium concentration ($[Ca^{2+}]_i$). U373 $\alpha_{1b}$ cells were labeled with fura-2AM. A, representative tracings from cells challenged with 1 $\mu$M $E_2$ (left), 100 $\mu$M noradrenaline (center) or $E_2$ (15 min) + NA (right) are shown. B, cells were challenged with different concentrations of NA. The maximum increase was considered as 100%. C, cells were incubated with different concentrations of $E_2$ during 15 min and then challenged with 100 $\mu$M NA. Mean values were plotted and vertical lines represent the S.E.M. of five experiments performed with different cell preparations.

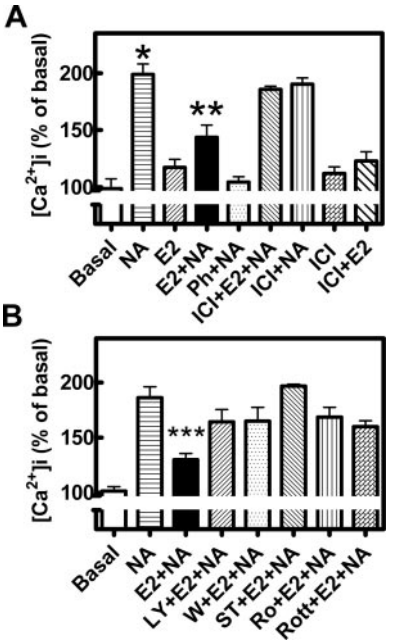


**Fig. 3.** Effect of 17$\beta$-estradiol ($E_2$) and noradrenaline (NA) on intracellular calcium concentration ($[Ca^{2+}]_i$). Roles of phosphoinositide-3-kinase and protein kinase C. A, cells were challenged with 100 $\mu$M NA, 1 $\mu$M $E_2$, 2 $\mu$M ICI 182,780, $E_2$ (15 min) + NA, ICI (10 min) + $E_2$, ICI (10 min) + NA and ICI (10 min) + $E_2$ (15 min) + NA. Phentolamine (Ph; 100 $\mu$M) ($\alpha_1$-AR antagonist) was used to verify the specificity of the response to NA. *, $p \leq 0.05$ versus all groups except ICI + $E_2$ + NA and ICI + NA. **, $p \leq 0.05$ versus all groups except $E_2$ and ICI + $E_2$. B, cells were challenged with 100 $\mu$M NA, 1 $\mu$M $E_2$ (15 min) + NA, 1 $\mu$M PI3K inhibitors: LY294002 (LY) or wortmanin (W) + $E_2$ + NA, 100 nM protein kinase C inhibitors: staurosporine (ST) or RO31-8220 (Ro) + $E_2$ + NA and finally with 100 nM protein kinase C $\delta$ inhibitor: rottlerin (Rott) + $E_2$ + NA. Inhibitors were added 10 min before NA or $E_2$. Plotted are the means ± S.E.M. of five experiments. ***, $p \leq 0.05$ versus all groups.

lecular events involved in these effects, associations of $\alpha_{1b}$-ARs with PI3K subunits and protein kinase Cδ were studied by coimmunoprecipitation. Association of $\alpha_{1b}$-ARs with PI3K-p85 and PI3K-p110 was detected under basal conditions and further increased after 17β-estradiol treatment (Fig. 6, A and C); ICI 182,780 inhibited the effect (Fig. 6, B and D). This action reached its maximum between 15 and 30 min after addition of estrogens. Consistent with other studies, ER-α and PI3K-p85 coimmunoprecipitated in the absence of stimulus, and this association further increased 5 to 30 min after 17β-estradiol treatment (Fig. 7A) (Simoncini et al., 2000; Segars and Diggers, 2002). Such association was inhibited by pretreatment with ICI 182,780 (Fig. 7B).

In agreement with previous data from our laboratory (Alcántara-Hernández et al., 2001), $\alpha_{1b}$-ARs coimmunoprecipitated with the α, δ, and ε isoforms of protein kinase C under nonstimulated conditions (data not shown). Interestingly, the only isoform whose coimmunoprecipitation increased in response to 17β-estradiol was protein kinase C δ; such association reached its maximum 15 to 30 min after treatment (Fig. 8A) and ICI 182,780 diminished the process (Fig. 8B). Attempts to detect coimmunoprecipitation of protein kinase C ζ with $\alpha_{1b}$-adrenoceptors were unsuccessful, although this isoform was easily detected in cell extracts (data not shown). Western blotting of the immunoprecipitated $\alpha_{1b}$-ARs indicated that loading did not vary in the coimmunoprecipitation studies and that the treatments did not alter their level (Supplemental Data 4).

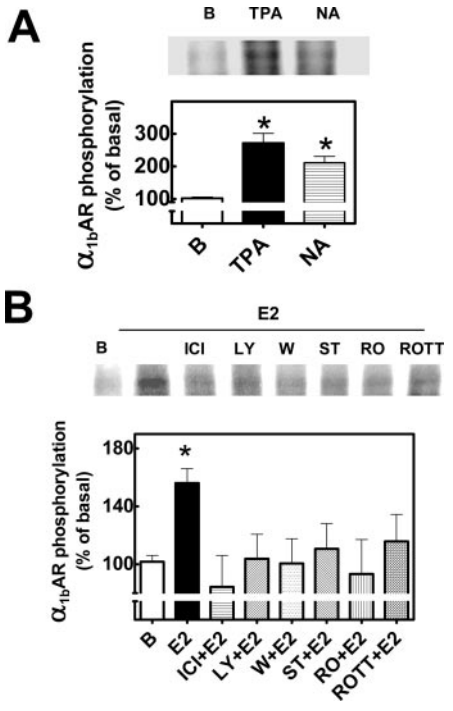


**Fig. 4.** Phosphorylation of $\alpha_{1b}$-ARs induced by 17β-estradiol ($E_2$), roles of PI3K and protein kinase C. U373 $\alpha_{1b}$ cells were metabolically labeled with [$^{32}$P]Pi and preincubated with 1 μM TPA or 100 μM noradrenaline (NA) (A) and without or with inhibitors (concentrations as in Fig. 3) (B) for 10 min and then challenged with 1 μM 17β-estradiol ($E_2$) for 30 min. Plotted are the means ± S.E.M. of five experiments. A representative autoradiograph is shown. *, $p \leq 0.05$ versus all groups.

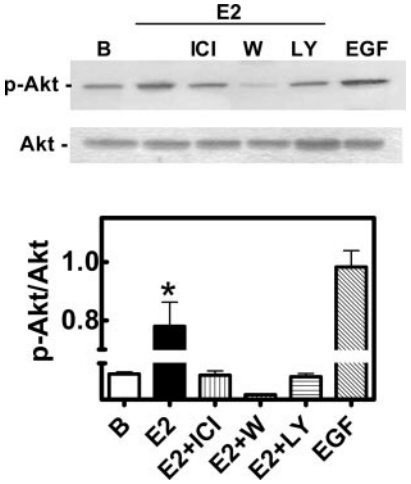


**Fig. 5.** 17-β Estradiol ($E_2$) effect on Akt phosphorylation. U373 $\alpha_{1b}$ cells were preincubated without or with inhibitors (concentrations as in Fig. 3) for 10 min and then challenged with 1 μM $E_2$ for 30 min. EGF (100 ng/ml) for 2 min was used as a positive control. Cells were lysed, proteins (70 μg) were separated by electrophoresis, electrotransferred and subjeted to Western blot analysis for *p*-Akt and total Akt. Representative Western blots for *p*-Akt and Akt are shown. Plotted are the means ± S.E.M. of the *p*-Akt/Akt ratios of five experiments performed using different cell preparations. *, $p \leq 0.05$ versus all groups.

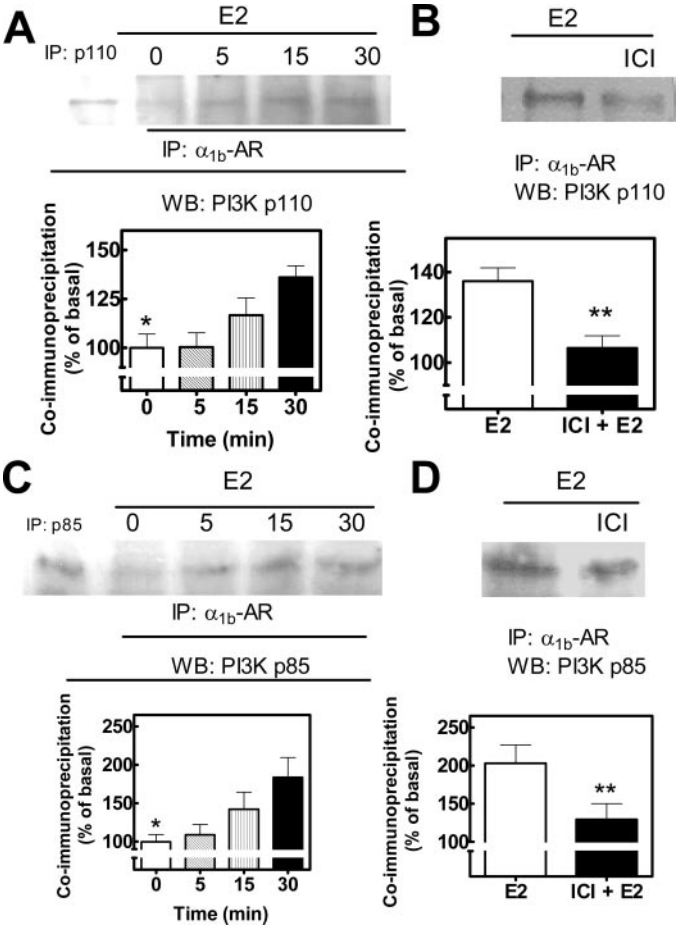


**Fig. 6.** Effect of 17β-estradiol ($E_2$) on $\alpha_{1b}$-AR and PI3K association. U373 $\alpha_{1b}$ cells were incubated with 1 μM $E_2$ at different times (A and C) or preincubated with or without 2 μM ICI for 10 min and then challenged with $E_2$ for 30 min (B and D). $\alpha_{1b}$-ARs were immunoprecipitated (IP) from lysed U373 $\alpha_{1b}$ cells and separated by electrophoresis. Western blots (WB) for PI3K p110 or PI3K p85 were carried out. Data are present as percentage of basal. Plotted are the mean ± S.E.M. of five experiments. *, $p \leq 0.05$ versus all other times. **, $p \leq 0.05$ versus $E_2$ alone. Representative blots are shown.

**Subcellular Localization of $\alpha_{1b}$-ARs Changes in Response to 17$\beta$-Estradiol.** Current concepts indicate that phosphorylation of G protein-coupled receptors is associated with receptor internalization (Pitcher et al., 1998; Ferguson, 2001). Therefore, our next aim was to test whether 17$\beta$-estradiol could affect the subcellular localization of these adrenoceptors. To study this, U373 MG WT cells were transfected with a plasmid that allowed the expression of human $\alpha_{1b}$-ARs fused to the EGFP, and fluorescence confocal microscopy was used. Figure 9A showed that $\alpha_{1b}$-ARs-EGFP were localized at the plasma membrane and to a lesser extent in intracellular vesicles; in contrast, when the EGFP alone was expressed it showed a homogenous cytoplasmic distribution.

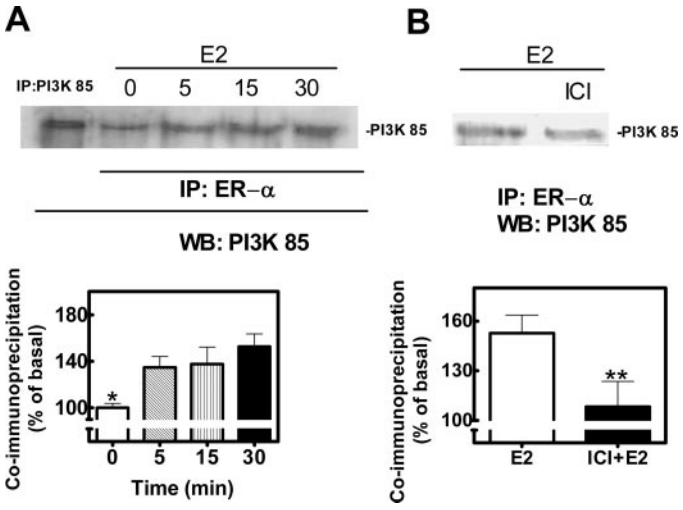


**Fig. 7.** Effect of 17$\beta$-estradiol ($E_2$) on ER-$\alpha$ and PI3K p85 association. U373 $\alpha_{1b}$ cells were incubated with 1 $\mu$M $E_2$ for the times indicated (A) or preincubated with or without 2 $\mu$M ICI for 10 min and challenged with $E_2$ for 30 min (B). ER-$\alpha$ was immunoprecipitated (IP) from lysed U373 $\alpha_{1b}$ cells. Immunoprecipitations were separated by electrophoresis and subjected to Western blot analysis (WB) for PI3K p85. Representative blots are shown. Data are presented as percentage of basal. Plotted are the mean ± S.E.M. of five experiments. *, $p \leq 0.05$ versus all groups. **, $p \leq 0.05$ versus $E_2$ alone.

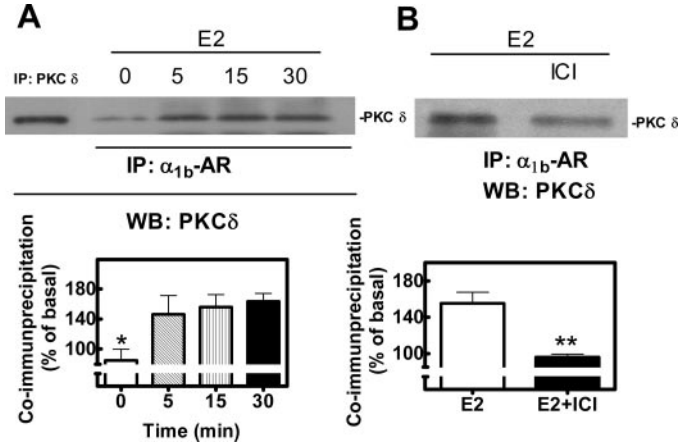


**Fig. 8.** Effect of 17$\beta$-estradiol ($E_2$) on the association of $\alpha_{1b}$-ARs and protein kinase C $\delta$ (PKC$\delta$). U373 $\alpha_{1b}$ cells were incubated with 1 $\mu$M $E_2$ at different times (A) or preincubated with or without 2 $\mu$M ICI for 10 min and challenged with $E_2$ for 30 min (B). $\alpha_{1b}$-ARs were immunoprecipitated (IP) from lysed U373 $\alpha_{1b}$ cells. Immunoprecipitations were separated by electrophoresis and subjected to Western blot analysis (WB) for protein kinase C $\delta$. Representative western blots are shown. Data are present as percent of basal. Plotted are the mean ± S.E.M. of five experiments. *, $p \leq 0.05$ versus all groups. **, $p \leq 0.05$ versus $E_2$ alone.

Treatment with 17$\beta$-estradiol induced $\alpha_{1b}$-AR internalization (Fig. 9A) and pretreatment with ICI 182,780 inhibited the process; EGFP distribution did not change with 17$\beta$-estradiol treatment (Fig. 9A). The internalization process was visualized in real time (see Supplemental Data 5), showing a relatively rapid effect of 17$\beta$-estradiol (15 min).

To demonstrate that $\alpha_{1b}$-AR-EGFP was functional, the $[Ca^{2+}]_i$ response was tested. In cells transfected with the $\alpha_{1b}$-ARs fused to the EGFP, noradrenaline clearly increased $[Ca^{2+}]_i$ and 17$\beta$-estradiol decreased the adrenergic effect in a fashion similar to that observed in cells transfected with $\alpha_{1b}$-ARs (Fig. 9B).

To confirm the intracellular localization of the adrenoceptors induced by 17$\beta$-estradiol, fluorescent markers were used. Figure 10 shows that after 17$\beta$-estradiol treatment, $\alpha_{1b}$-ARs were no longer colocalized with the FM-4-64 plasma membrane marker (top) but clearly colocalized with the intracellular membrane marker brefeldin A (bottom).

**ER-$\alpha$ Induces Desensitization, Phosphorylation, and Internalization of Human $\alpha_{1b}$-ARs.** To further test that the estrogen effects on desensitization and internalization were due to activation of ER-$\alpha$, HEK-293 cells were transiently transfected with human $\alpha_{1b}$-ARs fused to the enhanced green fluorescent protein. HEK-293 cells endogenously express a low amount of ER-$\alpha$ and the amount of ER-$\beta$ was below detection level (Fig. 11, top). It is noteworthy that, in these cells, 17$\beta$-estradiol did not alter the effect of NA. Therefore, we cotransfected $\alpha_{1b}$-ARs with ER-$\alpha$ to overexpress ER-$\alpha$ (Fig. 11, top). In cells cotransfected with both

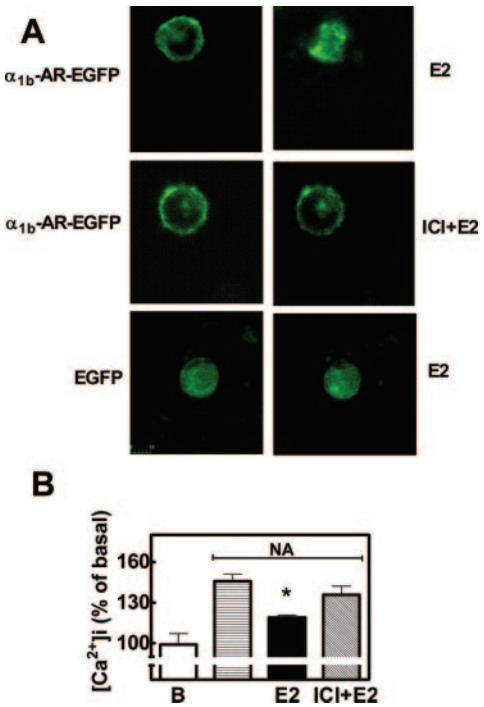


**Fig. 9.** Effect of 17$\beta$-estradiol ($E_2$) on $\alpha_{1b}$-AR internalization. U373 MG cells were transitorily transfected with the EGFP or the $\alpha_{1b}$AR-EGFP construction. Cells were treated with 1 $\mu$M $E_2$ (15 min) or with 2 $\mu$M ICI for 10 min and then challenged with $E_2$ for 15 min. A, EGFP was excited using the 488 nm line of a krypton/argon laser and the emitted fluorescence detected with a band-pass filter at 515 to 540 nm. Images are representative of three experiments using different cell preparations. B, $[Ca^{2+}]_i$ was determined in transfected cells loaded with fura 2-AM. Plotted are the means ± S.E.M. of three experiments. Data are present as percent of basal $[Ca^{2+}]_i$. *, $p \leq 0.05$ versus all groups.

receptors, 17$\beta$-estradiol diminished (~40%) the rise in $[Ca^{2+}]_i$ induced by noradrenaline (Fig. 11 lower). The effect of 17$\beta$-estradiol treatment (15 min) was abolished if cells were preincubated during 10 min with 2 $\mu$M ICI 182,780. In these cells, treatment with 17$\beta$-estradiol induced $\alpha_{1b}$-AR phosphorylation and internalization, and pretreatment with ICI 182,780 inhibited the process (Fig. 12).

## Discussion

Phosphorylation and heterologous desensitization of $\alpha_{1b}$-ARs has been previously observed in response to hormones and neurotransmitters acting though a variety of receptors; protein kinase C and PI3K play cardinal roles in these effects (Vázquez-Prado et al., 1997; Medina et al., 2000; Casas-González et al., 2003; García-Sáinz et al., 2004). Shedding of heparin binding-EGF and activation of EGF receptors seem to play a connecting role in the actions of G protein-coupled receptors. In the present article, phosphorylation and heterologous desensitization of $\alpha_{1b}$-ARs by activation of an intracellular receptor is presented. Our results suggest that estrogens stimulate PI3K activity through ER-$\alpha$, which leads to activation of protein kinase C $\delta$, phosphorylation of $\alpha_{1b}$-ARs, and internalization of these adrenoceptors.

It has been known for some time that formation of protein complexes is a central event in the signaling and regulation of receptors with intrinsic tyrosine kinase activity (Schlessinger, 2000). Nowadays, we know that this is a more general process not limited to such receptors (Pawson and Scott, 1997). Formation of multimolecular complexes has been shown to participate in the regulation of G protein-coupled receptors, including the $\beta_2$-adrenoceptors (Malbon et al., 2004) and the angiotensin $AT_1$ receptors (Olivares-Reyes et al., 2005), among others. The term signalplex has been coined to define such protein-protein complexes involved in signaling and receptor modulation (Neve, 2005). Protein-protein interactions are defining new connections in the signaling pathways potentially capable of controlling spatial and temporal responses in the cells (Pawson and Scott, 1997; Malbon et al., 2004). Our present data strongly suggest that the formation of a signalplex is involved in the modulation of $\alpha_{1b}$-ARs by estrogens. Such signalplex may involve $\alpha_{1b}$-ARs, PI3K and protein kinase C $\delta$. Whether such interaction may occur directly or via anchoring or scaffolding proteins remains unknown.

Defining how the actions of estrogens takes place is complicated by the fact that these steroids bind to and activate two intracellular receptors, and recent data have also shown the existence of membrane G protein-coupled receptors (GPR30) (Maggiolini et al., 2004; Revankar et al., 2005). Our present data clearly show the participation of ER-$\alpha$ in the modulation of $\alpha_{1b}$-ARs by 17$\beta$-estradiol. The barely detectable expression of ER-$\beta$ in U373 $\alpha_{1b}$ cells and the absence of detection in HEK-293 cells strongly suggests that this type of estrogen receptor did not participate in the effects described here. However, we cannot positively discard the participation of these intracellular receptors or of G protein-coupled estrogen receptors.

Different studies indicate that the rapid effects of estrogen are mediated by a subpopulation of the conventional estrogen receptors associated with the plasma membrane (Russell et al., 2000; Xu et al., 2003). ER-$\alpha$ fused to the green fluorescent protein has been visualized in the plasma membrane of neurites (Xu et al., 2003); in this work, ER-$\alpha$ was detected by Western blot analysis in membranes obtained from U373 cells (Supplemental Data 6).

The nongenomic effects of estrogens can involve different molecular mechanisms, among them the ER-$\alpha$ association to the p85$\alpha$ regulatory subunit of PI3K and activation of the PI3K/Akt signaling pathway (Lagrange et al., 1997; Simoncini et al., 2000; Segars and Driggers, 2002). Our results indicate that this process is involved in the modulation of $\alpha_{1b}$-ARs, which is consistent with previous findings from our laboratory (Vázquez-Prado et al., 1997; Medina et al., 1998; Casas-González et al., 2003; García-Sáinz et al., 2004). The dynamic association of PI3K with $\alpha_{1b}$-ARs in response to 17$\beta$-estradiol is consistent with a role in these effects. In addition, the ability of PI3K inhibitors to block the effects further suggests a role for this kinase. We used two inhibitors with different mechanisms of action; wortmannin is a potent and selective noncompetitive inhibitor of PI3K (Powis et al., 1994), whereas LY 294002 is an inhibitor that competes at the binding site for ATP of this kinase (Vlahos et al., 1994). These inhibitors clearly and consistently block $\alpha_{1b}$-AR desensitization and phosphorylation induced by 17$\beta$-estradiol.

PI3K is a modulator of protein kinase C activity through the synthesis of 3-phosphorylated phosphoinositides. These phospholipids can directly modulate the activity of protein kinase C. In addition, they are important modulators of the phosphoinositide-dependent protein kinase 1 (PDK-1), which phosphorylates the activation loop of some protein kinase C isoforms (Alessi et al., 1997; Le Good et al., 1998). In this

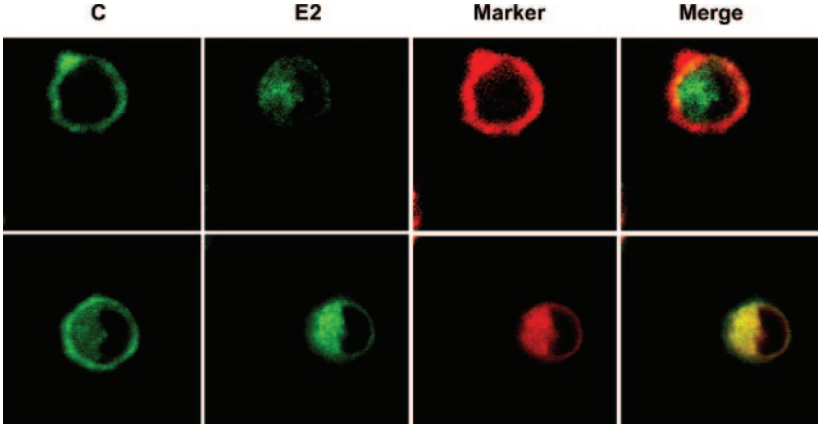


**Fig. 10.** $\alpha_{1b}$-AR intracellular localization. Fluorescent markers were used to confirm $\alpha_{1b}$-AR intracellular localization. $\alpha_{1b}$-AR-EGFP (green) localized in membrane was internalized by 1 $\mu$M 17$\beta$-estradiol treatment (15 min) ($E_2$). Plasma membrane marker FM-4–64 (top red and merge) and intracellular membrane systems marker brefeldin A (bottom red and merge) were used.

work, inhibitors of protein kinase C and PI3K block the effect of 17$\beta$-estradiol on desensitization and phosphorylation. The data suggest that these kinases may act in a sequence and not through independent pathways to phosphorylate $\alpha_{1b}$-ARs in response to 17$\beta$-estradiol. It is likely that activation of PI3K precedes that of protein kinase C.

It should be mentioned that the action of PI3K in mediating this effect might probably go beyond inducing protein kinase C activation. In a very elegant study, Naga Prasad et al. (2005) showed that PI3K plays a key role in $\beta_2$-AR endocytosis. PI3K is a dual kinase capable of phosphorylating phosphoinositides and proteins. It is noteworthy that the serine/threonine protein kinase activity of PI3K phosphorylates tropomyosin, and this action seems to be of cardinal importance for receptor internalization (Naga Prasad et al., 2005). It remains to be determined whether a similar role is played for internalization of $\alpha_{1b}$-ARs.

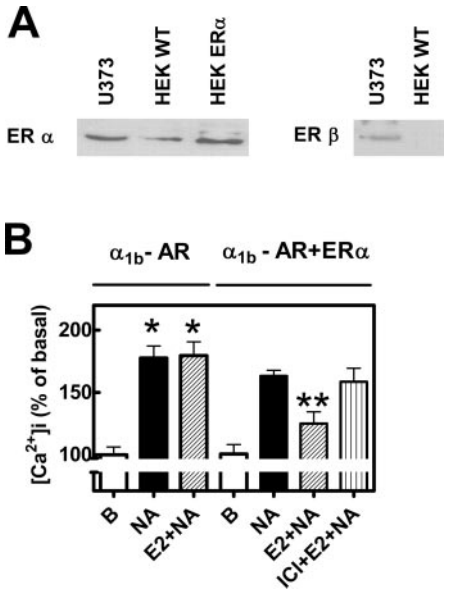


**Fig. 11.** Effect of ER-$\alpha$ activation on $\alpha_{1b}$-AR desensitization. A, lysates from U373 MG cells and HEK-293 cells, wild type or transfected with $\alpha_{1b}$-AR-EGFP and ER-$\alpha$, were separated by electrophoresis, and the gels were electrotransferred for Western blot detection of ER-$\alpha$ or ER-$\beta$. B, HEK-293 cells were transiently transfected with $\alpha_{1b}$-ARs fused to the enhanced green fluorescent protein and without or with human ER-$\alpha$ ($\alpha_{1b}$-AR+ ER-$\alpha$). $[Ca^{2+}]_i$ was determined in transfected cells challenged with 100 $\mu$M NA, 1 $\mu$M 17$\beta$-estradiol ($E_2$) (15 min) + NA, 2 $\mu$M ICI 182,780 + $E_2$ (15 min)+ NA. *, $p \leq 0.05$ versus basal (B), **, $p \leq 0.05$ versus all groups.

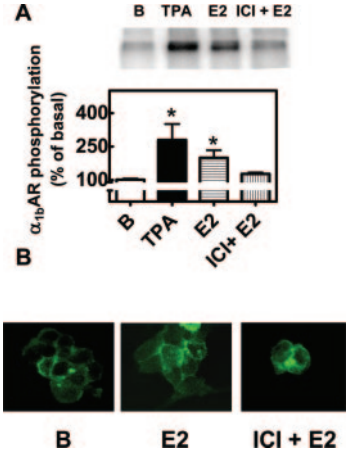


**Fig. 12.** Effect of ER-$\alpha$ activation on phosphorylation and internalization of $\alpha_{1b}$-AR. A, HEK-293 cells transfected with ER-$\alpha$ and $\alpha_{1b}$-AR-EGFP were metabolically labeled with $[^{32}P]P_i$ and incubated without any agent (B) or with 1 $\mu$M TPA (5 min), 1 $\mu$M 17$\beta$-estradiol ($E_2$) for 30 min or 2 $\mu$M ICI 182,780 for 15 min followed by $E_2$ (30 min). Plotted are the means ± S.E.M. of five experiments. A representative autoradiograph is shown. *, $p \leq 0.05$ versus B or $E_2$ + ICI. B, cells transfected with both receptors were fixed after treatment without or with 1 $\mu$M $E_2$ (15 min) or with 2 $\mu$M ICI for 10 min and then $E_2$ for 15 min. EGFP was excited using the 488 nm line of a krypton/argon laser and the emitted fluorescence detected with a band pass filter at 515 to 540 nm. Images are representative of three experiments.

Activation of protein kinase C is well known to block $\alpha_{1b}$-adrenoceptor actions and induce $\alpha_{1b}$-AR phosphorylation (García-Sáinz et al., 2000). Our group has observed that $\alpha$, $\delta$, and $\varepsilon$ isoforms of protein kinase C coimmunoprecipitated with $\alpha_{1b}$-ARs under basal conditions, and such association is dynamically increased by cell treatment with hormones that increase receptor phosphorylation (Alcántara-Hernández et al., 2001). In the present work, protein kinase C isoforms $\alpha$, $\delta$, and $\varepsilon$ coimmunoprecipitate with $\alpha_{1b}$-adrenoceptors in the basal state, but only protein kinase C $\delta$ increased this association with $\alpha_{1b}$-adrenoceptor after 17$\beta$-estradiol treatment. These data are consistent with the ability of rottlerin, a selective inhibitor of protein kinase C $\delta$, to block this effect. The data suggest that ER-$\alpha$, both subunits of PI3K, and protein kinase C $\delta$ may form a signalplex with $\alpha_{1b}$-ARs to induce regulation of this adrenoceptor.

Estrogens have been shown to modulate $\alpha_1$- and $\beta$-AR-mediated effects in rat pinealocytes (Hernandez-Diaz et al., 2001). In that work, 17$\beta$-estradiol treatment decreased $Ca^{2+}$ mobilization induced by activation of $\alpha_{1b}$-AR with noradrenaline, and the estrogen receptor antagonist ICI 182,780 abolished this effect. Although there is some similarity with our findings, these actions are probably unrelated, because these authors observed the effect only after a latency of 48 h, which suggests a genomic action of 17$\beta$-estradiol. Nevertheless, the data hint at the possibility that some genomic and nongenomic actions of estrogen lead to the same functional effect. Kelly and coworkers (Kelly and Wagner, 1999; Kelly et al., 2002) have shown that estrogen receptors can modulate the action of G protein-coupled receptors, such as the $\mu$-opioid and GABA-B receptors, by uncoupling the receptor to the rectifying $K^+$ channels through protein kinase A and protein kinase C.

The cardiovascular system is an important target tissue of estrogens and catecholamines. Estrogens have both short- and long-term effects protecting blood vessels by rapidly stimulating vasodilation through NO action (Edwards, 2005). Estrogens increase the expression of NO synthase and the rapid activation of the enzyme through the PI3K/Akt pathway (Lagrange et al., 1997; Simoncini et al., 2000; Segars and Driggers, 2002). One of the major actions of noradrenaline is to induce smooth muscle contraction, which participates in maintaining the vascular tone (García-Sáinz et al., 1999). It is possible that, through ER-$\alpha$, 17$\beta$-estradiol, besides its role in NO synthase action, could also regulate some of the physiological actions of noradrenaline in the cardiovascular systems.

We performed many of the present experiments using a neural cell line because it is well known that estrogens have neuroprotective properties (Lorenzo et al., 1992; Yu et al., 2004). Addition of 17$\beta$-estradiol increased the viability, survival, and differentiation of primary neuronal cultures. It has

been reported that estrogen-mediated neuroprotection in the retina involves the PI3K/Akt signal transduction pathway (Yu et al., 2004). Such neuroprotection might involve attenuation of receptor sensitivity as observed in the present work.

In summary, the present data show that, through ER-$\alpha$, 17$\beta$-estradiol induces $\alpha_{1b}$-AR phosphorylation, desensitization, and internalization. These results suggest the following working model: activated estrogen receptors associate with and stimulate PI3K activity; this leads to activation of protein kinase C $\delta$, which phosphorylates and desensitizes $\alpha_{1b}$-ARs. This process is accompanied by formation of a signalplex and receptor endocytosis.

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**Reference**

Alcántara-Hernández R, Leyva-Illades D, and García-Sáinz JA (2001) Protein kinase C-alpha(1b)-adrenoceptor coimmunoprecipitation: effect of hormones and phorbol myristate acetate. *Eur J Pharmacol* **419:**9–13.

Alessi DR, Deak M, Casamayor A, Caudwell FB, Morrice N, Norman DG, Gaffney P, Reese CB, MacDougall CN, Harbison D, et al. (1997) 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the *Drosophila* DSTPK61 kinase. *Curr Biol* **7:**776–789.

Arias-Montaño JA, Berger VA, Soria-Jasso LE, and Young JM (1999) Characterisation of alpha1B-adrenoceptors linked to inositol phosphate formation and calcium mobilisation in human astrocytoma U373 MG cells. *Naunyn-Schmiedeberg's Arch Pharmacol* **360:**533–539.

Casas-González P, Ruiz-Martínez A, and García-Sáinz JA (2003) Lysophosphatidic acid induces alpha-1b-adrenergic receptor phosphorylation through G-beta-gamma, phosphoinositide 3-kinase, protein kinase C and epidermal growth factor receptor transactivation. *Biochim Biophys Acta* **1633:**75–83.

Edwards DP (2005) Regulation of signal transduction pathways by estrogen and progesterone. *Annu Rev Physiol* **67:**335–376.

Ferguson SS (2001) Evolving concepts in G protein-coupled receptor endocytosis: the role in receptor desensitization and signaling. *Pharmacol Rev* **53:**1–24.

García-Sáinz JA, Rodríguez-Pérez CE and Romero-Ávila MT (2004) Human alpha-1D adrenoceptor phosphorylation and desensitization. *Biochem Pharmacol* **67:**1853–1858.

García-Sáinz JA, Vázquez-Prado J, and Medina LC (2000) Alpha 1-adrenoceptors: function and phosphorylation. *Eur J Pharmacol* **389:**1–12.

García-Sáinz JA, Vázquez-Prado J, and Villalobos-Molina R (1999) Alpha 1-adrenoceptors: subtypes, signaling and roles in health and disease. *Arch Med Res* **30:** 449–458.

Grynkiewicz G, Poenie M, and Tsien RY (1985) A new generation of $Ca^{2+}$ indicators with greatly improved fluorescence properties. *J Biol Chem* **260:**3440–3450.

Hernandez-Diaz FJ, Sanchez JJ, Abreu P, Lopez-Coviella I, Tabares L, Prieto L, and Alonso R (2001) Estrogen modulates alpha(1)/beta-adrenoceptor- induced signaling and melatonin production in female rat pinealocytes. *Neuroendocrinology* **73:**111–122.

Kelly MJ, Qiu J, Wagner EJ, and Ronnekleiv OK (2002) Rapid effects of estrogen on G protein-coupled receptor activation of potassium channels in the central nervous system (CNS). *J Steroid Biochem Mol Biol* **83:**187–193.

Kelly MJ and Wagner EJ (1999) Estrogen Modulation of G-protein-coupled Receptors. *Trends Endocrinol Metab* **10:**369–374.

Koike S, Sakai M, and Muramatsu M (1987) Molecular cloning and characterization of rat estrogen receptor cDNA. *Nucleic Acids Res* **15:**2499–2513.

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, and Gustafsson JA (1996) Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci USA* **93:**5925–5930.

Lagrange AH, Ronnekleiv OK, and Kelly MJ (1997) Modulation of G protein-coupled receptors by an estrogen receptor that activates protein kinase A. *Mol Pharmacol* **51:**605–612.

Le Good JA, Ziegler WH, Parekh DB, Alessi DR, Cohen P, and Parker PJ (1998) Protein kinase C isotypes controlled by phosphoinositide 3-kinase through the protein kinase PDK1. *Science (Wash DC)* **281:**2042–2045.

Lorenzo A, Diaz H, Carrer H, and Caceres A (1992) Amygdala neurons in vitro: neurite growth and effects of estradiol. *J Neurosci Res* **33:**418–435.

Maggiolini M, Vivacqua A, Fasanella G, Recchia AG, Sisci D, Pezzi V, Montanaro D, Musti AM, Picard D, and Ando S (2004) The G protein-coupled receptor GPR30 mediates c-fos up-regulation by 17$\beta$-estradiol and phytoestrogens in breast cancer cells. *J Biol Chem* **279:**27008–27016.

Malbon CC, Tao J, and Wang HY (2004) AKAPs (A-kinase anchoring proteins) and molecules that compose their G-protein-coupled receptor signalling complexes. *Biochem J* **379:**1–9.

Medina LC, Vázquez-Prado J, and García-Sáinz JA (2000) Cross-talk between receptors with intrinsic tyrosine kinase activity and alpha1b-adrenoceptors. *Biochem J* **350:**413–419.

Medina LC, Vázquez-Prado J, Torres-Padilla ME, Mendoza-Mendoza A, Cruz Muñoz ME, and García-Sáinz JA (1998) Crosstalk: phosphorylation of alpha1b-adrenoceptors induced through activation of bradykinin B2 receptors. *FEBS Lett* **422:**141–145.

Naga Prasad SV, Jayatilleke A, Madamanchi A, and Rockman HA (2005) Protein kinase activity of phosphoinositide 3-kinase regulates beta-adrenergic receptor endocytosis. *Nat Cell Biol* **7:**785–796.

Neve KA (2005) Double feature at the signalplex. *Mol Pharmacol* **68:**275–278.

Olivares-Reyes JA, Shah BH, Hernandez-Aranda J, Garcia-Caballero A, Farshori MP, Garcia-Sainz JA, and Catt KJ (2005) Agonist-induced interactions between angiotensin AT1 and epidermal growth factor receptors. *Mol Pharmacol* **68:**356–364.

Pawson T and Scott JD (1997) Signaling through scaffold, anchoring and adaptor proteins. *Science (Wash DC)* **278:**2075–2080.

Penn RB, Pronin AN, and Benovic JL (2000) Regulation of G protein-coupled receptor kinases. *Trends Cardiovasc Med* **10:**81–89.

Pitcher JA, Freedman NJ, and Lefkowitz RJ (1998) G protein-coupled receptor kinases. *Annu Rev Biochem* **67:**653–692.

Pitcher JA, Payne ES, Csortos C, DePaoli-Roach AA, and Lefkowitz RJ (1995) The G-protein-coupled receptor phosphatase: a protein phosphatase type 2A with a distinct subcellular distribution and substrate specificity. *Proc Natl Acad Sci USA* **92:**8343–8347.

Powis G, Bonjouklian R, Berggren MM, Gallegos A, Abraham R, Ashendel C, Zalkow L, Matter WF, Dodge J, Grindey G, et al. (1994) Wortmannin, a potent and selective inhibitor of phosphatidylinositol-3-kinase. *Cancer Res* **54:**2419–2423.

Revankar CM, Cimino DF, Sklar LA, Arterburn JB, and Prossnitz ER (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science (Wash DC)* **307:**1625–1630.

Russell KS, Haynes MP, Sinha D, Clerisme E, and Bender JR (2000) Human vascular endothelial cells contain membrane binding sites for estradiol, which mediate rapid intracellular signaling. *Proc Natl Acad Sci USA* **97:**5930–5935.

Schlessinger J (2000) Cell signaling by receptor tyrosine kinases. *Cell* **103:**211–225.

Segars JH and Driggers PH (2002) Estrogen action and cytoplasmic signaling cascades. Part I: membrane-associated signaling complexes. *Trends Endocrinol Metab* **13:**349–354.

Simoncini T, Hafezi-Moghadam A, Brazil DP, Ley K, Chin WW, and Liao JK (2000) Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature (Lond)* **407**(6803)**:**538–541.

Strom A, Hartman J, Foster JS, Kietz S, Wimalasena J, and Gustafsson JA (2004) Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. *Proc Natl Acad Sci USA* **101:**1566–1571.

Vázquez-Prado J, Medina LC, and García-Sáinz JA (1997) Activation of endothelin ETA receptors induces phosphorylation of $\alpha_{1b}$-adrenoreceptors in Rat-1 fibroblasts. *J Biol Chem* **272:**27330–27337.

Vlahos CJ, Matter WF, Hui KY, and Brown RF (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *J Biol Chem* **269:**5241–5248.

Xu Y, Traystman RJ, Hurn PD, and Wang MM (2003) Neurite-localized estrogen receptor-alpha mediates rapid signaling by estrogen. *J Neurosci Res* **74:**1–11.

Yu X, Rajala RV, McGinnis JF, Li F, Anderson RE, Yan X, Li S, Elias RV, Knapp RR, Zhou X, et al. (2004) Involvement of insulin/phosphoinositide 3-kinase/Akt signal pathway in 17$\beta$-estradiol-mediated neuroprotection. *J Biol Chem* **279:**13086–13094.

**Address correspondence to:** J. Adolfo García-Sáinz, Instituto de Fisiología Celular, UNAM, Ap. postal 70–248, México D. F. 04510. E-mail: agarcia@ifc.unam.mx