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Short-Term Activation by low 17β-Estradiol Concentrations of the Na^+/H^+ Exchanger in Rat Aortic Smooth Muscle Cells: Physiopathological Implications

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ABSTRACT

Low physiological concentrations of 17β-estradiol increased the intracellular pH of rat aortic smooth muscle cells by a rapid nongenomic mechanism. This effect was due to stimulation of the Na^+/H^+ exchanger activity, measured using the intracellular pH-sensitive fluorescent probe 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein. The 17β-estradiol gave rise to a bell-shaped dose-response, with a maximum at 10^{-12} M and no significant effect at 10^{-9} M. The specificity of the effect was verified by the use of the Na^+/H^+ exchanger inhibitor 5-(ethyl-N-isopropyl)amiloride and by the lack of effect of the isomer 17α-estradiol. Inhibitors of the nuclear estrogen receptors, tamoxifen and ICI 182,780, completely prevented activation of the exchanger by 17β-estradiol. The effect of low estrogen concentrations on the intracellular pH was mimicked by both norepinephrine and phenylephrine, suggesting a connection between the increase of intracellular pH and the muscle contraction process. The transduction mechanism for this nongenomic effect of estrogens did not involve modulation of the cAMP content, whereas inositol 1,4,5-trisphosphate, protein kinase C and MAP kinase pathways appear to play a role, as indicated by both pharmacological approaches and immunoblot experiments on protein kinase C translocation and ERK phosphorylation. These results for the first time provide evidence for a nongenomic effect of low physiological concentrations of 17β-estradiol on intracellular pH, that, together with other factors, may contribute to the development of hypertension and atherosclerosis in men and postmenopausal women and increase the risk of cardiovascular disease.

Introduction

Estrogens modulate a wide variety of cellular effects, acting both at the intracellular level and on the plasma membrane. In the classical model estrogens bind to the nuclear receptors ERα and ERβ whose molecular characteristics have been well established, acting as ligand-dependent transcription factors to regulate gene expression and protein synthesis (1). The membrane actions of estrogens are believed to be triggered after hormone binding to plasma membrane receptors, giving rise to nongenomic effects characterized by a rapid onset of action, within seconds to minutes (2, 3), but the nature of the putative membrane estrogen receptor is still debated. It is not yet clear whether this membrane receptor is similar or even identical to the nuclear ERs or if it has a completely different structure (4, 5); the presence of a membrane receptor unrelated to both ERα and ERβ has been postulated and defined as γ-adrenergic receptor (6).

The estradiol-induced nongenomic responses observed seem to depend on the type of cell and tissue, and the signaling mechanisms involved appear to be similar to those evoked by the most common growth factors and hormones acting through membrane receptors: intracellular [Ca^{2+}], PKC, PLC, cAMP, the MAPK pathway, and intracellular pH (7-9). At present it is completely unknown which genomic or non genomic mechanisms are responsible for the vascular response to estrogen influence. It is generally accepted that estrogens have direct acute vasodilatory effects on vascular tone, brought about through both endothelium-independent (10) and endothelium-dependent mechanisms (11), and that this is part of the cardioprotective action of estrogens in premenopausal women (12). Some specific functions of vascular smooth muscle cells such as contraction, proliferation, production and composition of the extracellular matrix have been shown to be affected by estrogens. A decreased contractile response of rat tail artery to norepinephrine, arginine-vasopressin and KCl (13) and of isolated aorta to α-adrenergic stimulation has been observed after exposure to

high concentrations of estradiol (14). The estradiol effect in the physiological to supraphysiological concentration range on smooth muscle contraction has been ascribed to different mechanisms: the blocking of Ca^{2+} channels and inhibition of Ca^{2+} influx, the activation of calcium- or voltage-activated K^+-channels and to changes in cAMP and cGMP levels (15). On the other hand, the estrogen-induced endothelium-dependent relaxation was commonly associated with the stimulation of NO production from the vascular endothelium (16). While many data are available on the high estradiol concentration effects, the lower concentrations corresponding to the postmenopausal or masculine levels have been less investigated. Epidemiological studies have shown significant differences between males and females in the onset of osteoporosis or cardiovascular disease. In the males the risk is constantly growing with the age, while premenopausal women show a lower occurrence attributed to the presence of estrogens, in postmenopausal women, when the estrogens level is low, the risk become comparable to that of men.

It could therefore be of high physiological relevance to know the mechanisms taking place when estrogens fall to postmenopausal levels and the protective effect is lost in cells which are strongly affected by estrogens such as aortic smooth muscle cells. Taking into account that the physiological concentration range of estrogens can be very wide, going from pregnancy (10^{-9}- 10^{-8} M) to postmenopausal (10^{-12} - 10^{-10} M) we will call the first range 'high physiological' and the second range 'low physiological' throughout this paper (17).

A quite complex relationship has been reported in smooth muscle between intracellular pH and the contraction/relaxation process. Changes in intracellular pH have been found to produce marked effects on the contraction of smooth muscles. In general, muscle tension increases as intracellular pH increases, even though some smooth muscles can produce more force during an intracellular acidification as it happens in the pulmonary artery (18). The intracellular pH is regulated by the Na^+/H^+ antiport, a plasma membrane protein

exchanging Na^+ and H^+ ions according to the concentration gradient; besides its housekeeping function of regulation of intracellular pH and cell volume, this protein is nowadays considered also to play a more regulatory role in the cell (19). In particular, an increase of intracellular pH due to activation of the Na^+/H^+ exchanger by different kinases can represent the first response of the cell to a wide range of physiological (hormones and growth factors; 20-22) and pathological signals (oxidative stress, ischemia, inflammatory cytokines; 23-27). Thyroid hormones are best known for exerting their physiological action through intracellular receptors and genomic long-term mechanism, in analogy with estrogens, they can also give rise to short-term nongenomic effects with a time-course of seconds to minutes. Among these effects is a fast increase in intracellular pH due to activation of the Na^+/H^+ antiport in L-6 myoblasts from rat skeletal muscle (21). Nongenomic thyroid hormone stimulation of the Na^+/H^+ exchanger and of the amino acid transport, System A, was recently shown in chick embryo hepatocytes and was found to be mediated by a signal transduction pathway involving protein kinase C (PKC), the mitogen-activated protein kinase (MAPK) pathway and phosphatidylinositol-3 kinase (22). However, short-term effects on the Na^+/H^+ exchanger have also been reported for aldosterone by a rapid nongenomic mechanism in Human Mononuclear Cells and vascular smooth muscle cells (2). Furthermore, 17β-estradiol (10^{-8} M) is able to activate, by a mechanism not sensitive to cycloheximide, a regulatory factor for the Na^+/H^+ exchanger (NHE-RF), involved in the inhibition of the Na^+/H^+ exchanger by the PKA-cAMP-dependent mechanism in the renal tubule (28). These findings suggest that both estrogens and thyroid hormones may give rise to nongenomic effects through modulation of the exchanger activity. Taking into account the role played by intracellular pH in the contraction/relaxation process of smooth muscle, we have studied whether estrogens modulate the activity of the exchanger in RASM cells. Our data show that 17β-estradiol, in a low physiological concentration range (10^{-12}-10^{-10} M) increases intracellular pH in rat aortic

smooth muscle cells, by activation of the Na^+/H^+ exchanger, with a nongenomic mechanism involving both protein kinase C and MAPK pathways. We discuss the potential physiopathological implications of the observed effects at these low hormone concentrations.

Materials and Methods

Cell culture

Rat Aorta Smooth Muscle (RASM) cells were prepared from explants of thoracic aorta of 15-week-old male and female Wistar rats by the method of Ross (29). Cells were cultured in 25 cm^2 plastic tissue flasks and grown in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% FCS, 100 µg/ml streptomycin and 100 U/ml penicillin, in an atmosphere of 5% CO_2 at 37 °C. RASM cells were harvested once a week by treatment with 0.25% trypsin and refed every 2 days. For the experiments cells were used at passages 3 –10 as confluent monolayers after 6-8 days from seeding.

Cell growth was measured on cells seeded in 60 x 15 mm petri dishes 1.0-1.5 × 10^5 cells/well and grown in DMEM supplemented as reported above, in the presence and absence of different concentrations of hormones. The medium with or without hormones was changed every 24 hours. Cells were harvested after mild trypsinization and counted in a Neubauer chamber. These studies were conducted in accordance with the directives of the European Community (86/609/EEC) on the care and use of laboratory animals.

Determination of intracellular pH

For the experiments of fluorescence assays, cells were grown in chamber slides (Lab-Tek, Nunc, Naperville, IL) and used at confluency. Before the experiment cells were rendered quiescent by serum deprivation for 5 hours.

Intracellular pH was measured by the fluorescent intracellular pH indicator 2',7'-bis-(2-carboxyethyl)-5(6)-carboxyfluorescein tetra-acetoxymethyl ester (BCECF/AM). To rule out the contribution of HCO_3^--dependent transport mechanisms (30), all experiments were carried out in bicarbonate-free buffer with the following composition (mM): 135 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 20 HEPES, pH 7.3. This buffer (henceforth called Na^+

buffer) was used for the incubation with the fluorescent probe and for the determination of intracellular pH unless otherwise stated; the cells incubated in this buffer were considered virtually depleted of bicarbonate.

Incubation with the fluorescent dye was carried out as follows: cells were washed twice with Na^+ buffer and were thus considered bicarbonate-free. Cells were then incubated in Na^+ buffer with the fluorescent dye (1 mg/ml in dimethyl sulfoxide) at the final concentration of 1 μg/10^6 cells, for 10 min at 37 °C in the dark. Then the medium containing the dye was eliminated, and the cells were washed twice with the Na^+ buffer.

Routinely at the end of each experiment, calibration of fluorescence *vs* pH was carried out by an established method (31) using the K^+-H^+ ionophore nigericin (5 μM) added to cells suspended in a potassium solution having the same composition as Na^+ buffer, but with NaCl substituted by equimolar concentrations of KCl. Under these conditions, intracellular and extracellular pH are equilibrated ($pH_i = pH_o$). The extracellular pH was changed with 10-μl aliquots of 1 M 2-[N-morpholino] ethanesulfonic acid (MES) or 1 M Tris(hydroxymethyl)aminomethane (Tris) and determined with a glass electrode inserted directly into the cuvette. Intracellular fluorescence was determined and plotted *vs* extracellular pH. The calibration curve was linear in the pH range 6.5 - 7.8 (not shown).

Fluorescence was measured under continuous magnetic stirring at a controlled temperature (37°C) in a Perkin Elmer LS-50B luminescence spectrometer equipped with a fast filter accessory for the dual excitation single emission ratio technique. Excitation wavelengths were set at 498 nm (pH-dependent component) and 450 nm (pH-independent component) with emission at 530 nm, using 5 and 10 nm slits, respectively, for the two light paths. This allowed measurements of intracelluar pH that were not depending on cell concentration and dye loading (32).

Inositol polyphosphate production.

Subconfluent cells were exposed for 24 h to 2-[^{3}H]myoinositol (1 μCi/ml; sp.act. 20 C_i/mmol). The medium was discarded and, after three washes with phosphate buffered saline (PBS), substituted with serum-free fresh medium containing 17β-estradiol (10^{-8} M) or vehicle (dimethyl sulfoxide/PBS 1/100 v/v). In some experiments ICI 182,780 (1 μM) was added 15 min before estradiol treatment.

At the end of the incubation cells were rinsed with PBS and scraped with 1 ml TCA 10% containing 2 mM EDTA. The TCA soluble fraction was washed with diethyl ether and analysed by anionic exchange chromatography on Dowex 1X-8 resin, formate form. Free inositol, inositol monophosphate, inositol bisphosphate, inositol trisphosphate and inositol tetrakisphosphate were eluted from small glass columns with water, 0.2, 0.4, 0.8 and 1,0 M ammonium formate in formic acid (0.1 M) respectively (33) and the radioactivity was measured by a liquid scintillation counter (Packard, Downers Grove, IL). Under these conditions, a [^{3}H]IP_3 standard was completely eluted from columns with 15 ml 0.8 M ammonium formate in formic acid (0.1 M). The TCA insoluble fraction was dissolved in 1 M NaOH and the protein content was measured (34). Inositol(1,4,5)P_3 and cAMP levels in the cells were determined by radioligand assay kits.

Western blot analysis

Subconfluent cells after hormone treatment were washed twice in ice-cold PBS and then scraped into ice-cold 10 mM Tris-HCl pH 7.4 containing 1 mM $CaCl_2$, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride and 5 μg/ml leupeptin. After sonication, soluble and particulate fractions were obtained by centrifuging samples at 100,000 × g for 30 min. Total cell lysate were obtained as already described (9, 33). Proteins were solubilised in 0.125 M Tris-HCl pH 6.8 containing 10% SDS, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml

leupeptin, and boiled for 2 min. Equal amounts (20 μg) of cytosol, membrane and total proteins were separated by 7% SDS-PAGE (PKC-α) or 10% SDS-PAGE (ERK and ERK-P) at 40 mA for 1 h, and then transferred to nitrocellulose filters. Filters were saturated with 1% bovine serum albumin and probed at room temperature for 1 h with monoclonal anti-PKC-α or anti-ERK or anti-ERK-P antibodies (1 μg/ml). Bound antibodies were visualised using enhanced chemiluminescence detection (CDP-Star, NEN Boston, MA). Protein content was quantified by densitometry analysis of Western blots (Fluor-S, Biorad, Hercules, CA).

Solutions

BCECF/AM (1 mg/ml), 5-(N-ethyl-N-isopropyl)amiloride (EIPA; 10 mM), genistein, U 73122, H-7, and calphostin C were dissolved in dimethyl sulfoxide, which did not affect the fluorescence signal. Nigericin (10 mM) and 17β-estradiol 3-benzoate (1,3,5[10]-estratriene-3,17β-diol; 1 mM), 17α-estradiol (1,3,5 [10]-estratriene-3,17α-diol), tamoxifen, ICI 182,780 and neomycin were dissolved in ethanol. Norepinephrine and phenylephrine were aqueous solutions.

Materials

DMEM, antibiotics, and sterile plasticware for cell culture were from Flow Laboratory (Irvine, UK). FBS was from GIBCO (Grand Island, NY). BCECF/AM was obtained from Molecular Probes (Eugene, OR). [^{3}H]-cAMP and [^{3}H]IP_3 assay kits were from Amersham (Freiburg, Germany). Nigericin, HEPES, MES, Tris, 17α-estradiol, 17β-estradiol, genistein, H-7, neomycin, norepinephrine, phenylephrine, BAPTA/AM, and Dowex 1X-8 resin were from Sigma (St. Louis, MO). 5-(Ethyl-N-isopropyl)amiloride (EIPA) was obtained from Research Biochemicals International (Natick, MA). PD 98059, U 0126 were from Alexis Biochemicals (Laufelfingen, Switzerland). Tamoxifen and ICI 182,780 were from Tocris

Cookson (Bristol, UK). U 73122 and RO 31-8220 were obtained from Calbiochem (La Jolla, CA). Calphostin C was from ICN Pharmaceuticals (Costa Mesa, CA). Monoclonal anti PKC-α or anti-ERK or anti-ERK-P antibodies were provided from Santa Cruz Biotechnology (Santa Cruz, CA). All other chemicals were of the purest grade available from Merck (Darmstadt, Germany).

Results

Effect of 17β-estradiol on cell proliferation

In order to verify the inhibitory effect of estrogens on cell growth, considered to be part of the cardioprotective actions of these hormones in premenopausal women, the dose-response of 17β-estradiol on RASM cell proliferation was determined over a wide concentration range (Fig.1). The hormone showed antiproliferative properties after 72 hours, corresponding to the most active period of exponential cell growth. The effect was only significant in the 10^{-10}-10^{-8} M concentration range, indicating that the antiproliferative properties, which were previously observed with high physiological and pharmacological concentrations in the same cells (35-37), are not present at low physiological concentrations of the hormone.

Effect of 17β-estradiol on the intracellular pH at the steady state.

In the standard bicarbonate-free Na^+ buffer solution, the mean steady-state pH_i of rat aortic smooth muscle cells in culture was 7.00 ± 0.04 (mean ± SD; N=20) in agreement with earlier reported data (24).

Addition of very low concentrations of 17β-estradiol, 10^{-12} - 10^{-10} M, resulted in a lasting increase in the intracellular pH due to the stimulation of the Na^+/H^+ exchanger. The maximum increase at equilibrium was 0.18 ± 0.04 ΔpH/40 min over the basal value (mean ± S.D.; n =12) and was found at 10^{-12} M 17β-estradiol, whereas lower effects were obtained at higher and lower hormone concentration, as reflected by the bell-shaped dose-response curve (Fig. 2 upper panel). The time course of the effect of 10^{-12} M estradiol showed that the intracellular pH started to increase immediately after hormone addition and the maximum was reached within about 30-40 min, a slightly longer time than that normally required for most hormones or growth factors to activate the Na^+/H^+ exchanger (Fig. 2 lower panel). The effect

was specific since it was inhibited by 10 μM EIPA, a derivative of amiloride and a specific inhibitor of this transport system (Fig. 2 lower panel).

The stereospecificity of the stimulation obtained with 17β-estradiol was evaluated by the use of 17α-estradiol. This isomer was tested over a wide range of concentrations, but no significant effect was observed on the intracellular pH; the result obtained with one representative concentration (10^{-12} M) is reported in Fig. 3. The same figure shows that treatment with the most common inhibitors of the nuclear estrogen receptor, ICI 182,780 and tamoxifen, completely prevented the effect of 17β-estradiol on the intracellular pH. Taken together these results confirm the presence of an estrogen receptor with similar binding site topology as the nuclear receptor types.

Norepinephrine and phenylephrine mimic the nongenomic activation of the Na^+/H^+ exchanger by 17β-estradiol

It is known that changes of pH can alter the force development in smooth muscle affecting various aspects of muscle contraction such as the rate of myosin ATPase activity, the sensitivity of the contractile units to Ca^{2+} and many others (18). The sensitivity of myofilaments to Ca^{2+} is pH dependent, and in general tension falls when pH falls. Estrogens in the range 10^{-6}-10^{-7} M give rise to vasodilation of smooth muscle by a rapid nongenomic mechanism involving membrane-based phenomena, such as alteration of membrane ionic permeability and activation of membrane-bound enzymes, even though the classical genomic effects must be also taken into account (38, 39). In order to find a correlation between the increase of intracellular pH by 17β-estradiol and vascular tone, experiments were carried out with α-adrenergic agonists for smooth muscle.

Norepinephrine and phenylephrine increased intracellular pH by activating the Na^+/H^+ exchanger with a time-course similar to that showed by 17β-estradiol, and again the effect

was blocked by EIPA, indicating the specificity of these agonists (Fig. 4). The addition of an α-adrenergic agonist together with 17β-estradiol did not give rise to any additive response or inhibition of the intracellular pH increase, suggesting the possibility of a common signal transduction pathway (not shown). All experiments involving the α-adrenergic agonists to be reported in the following were carried out with both norepinephrine and phenylephrine, but only the data obtained with phenylephrine will be shown for the sake of brevity.

Transduction pathway for the short-term effect of 17β-estradiol and phenylephrine on intracellular pH

To identify the transduction pathway involved in the activation of the Na^+/H^+ exchanger by 17β-estradiol in RASM cells we assessed the production of second messengers, as well as the involvement of kinases and phospholipases, by means of a pharmacological approach, and using the Western blotting technique. The tyrosine kinase inhibitor genistein, the PLC inhibitors neomycin and U 73122, the protein kinase C inhibitors H-7 and calphostin C, and the MAPK pathway inhibitors PD 98059 and U 0126 were all able to prevent the activation of the Na^+/H^+ exchanger by estrogens (Fig. 5). These results suggest a transduction mechanism involving all these enzymes. To verify whether activation of the Na^+/H^+ exchanger by α-adrenergic agonists utilised similar pathways, the involvement of PKC and MAP kinase was probed by the use of inhibitors. All compounds used were effective in the inhibition of the Na^+/H^+ exchanger activity by phenylephrine too (Fig. 6).

Inhibitors of signal transduction pathways often turn out to be less specific than assumed, and studies on activation mechanisms should not be based only on the effects observed with inhibitors. Further experiments were therefore made to verify the role of key compounds of the phosphoinositide and MAPK pathways. 17β-Estradiol stimulated the production of IP_3, the second messenger derived from phospholipase C catalysed hydrolysis,

as reflected in the higher steady state level of IP_3 (Fig. 7A). The increase in IP_3 production, measured as incorporation of ^{3}H-myoinositol was rapid, detectable after 1 min, well in agreement with previously published results (40) and with a maximum at the hormone concentration of 10^{-10} M (Fig 7 B). Inhibition of the IP_3 increase by ICI 182,780 and U 73122 indicated an ER-dependent involvement in PLC activation (Fig. 7 C).

The sharp increase in IP_3 production prompted us to evaluate the role of intracellular calcium in the mediation of nongenomic effects of estrogens on the intracellular pH. For that purpose we used BAPTA, a selective chelator of intracellular Ca^{2+} (41). Experiments were carried out on RASM cells preincubated with BAPTA/AM (25 μM), a hydrophobic form of BAPTA, that enters the cell and then becomes deesterified to BAPTA and trapped within. BAPTA/AM pretreatment completely prevented the effect of both estrogens and α-adrenergic agonists (Fig. 8). Same result was found by chelating extracellular calcium with 5 μM EGTA (not shown).

The dose-response of estrogen effect on the translocation of PKC-α from the cytosol to the membrane showed a major effect in the concentration range of 10^{-12}-10^{-10} M (Fig. 9 panel A). ICI 182,780 and U 73122 (Fig. 9 panel B) fully inhibited the translocation even at the most effective estrogen concentration (10^{-10} M), confirming a receptor-dependent involvement of PLC activation. As to the MAPK pathway, our data show that the level of phosphorylation of ERK2 induced by estrogen was increased in the range 10^{-14}-10^{-8} M, indicating the ability of 17β-estradiol to activate this enzyme (Fig. 10 panel A). The same figure also shows the effect of inhibitors ICI 182,780, RO 31-8220, and U 0126 on the activation of the MAPK pathway by 17β-estradiol. The ICI inhibition and the ineffectiveness of the PKC inhibitor, RO 31-8220, on ERK2 phosphorylation supports the involvement of an estrogen receptor and the IP_3/PKC-α independence of this activation (Fig. 10 panel B).

It has been reported that estrogens regulate the expression of NHE-RF, which is involved in the inhibition of the Na^+/H^+ exchanger in the renal tubule through a nongenomic mechanism depending on cAMP and protein kinase A activation (28). In our cells, 17β-estradiol did not give rise to any modulation of the cAMP levels, thus ruling out the contribution of cAMP to the activity of the Na^+/H^+ exchanger activity by estrogens in this experimental system (Fig. 11).

Discussion

In this paper we report for the first time that 17β-estradiol, in the low physiological concentration range (10^{-12}-10^{-10} M) close to that found in men or postmenopausal women (42), increases intracellular pH of rat aortic smooth muscle cells in culture. The effect on intracellular pH is due to the specific activation of the Na^+/H^+ exchanger, since it is prevented by the inhibitor EIPA and since the stereoisomer 17α-estradiol is totally ineffective. The rapid onset of the intracellular pH response suggests that this effect cannot be explained by a classical genomic mechanism, and must be considered nongenomic. The hormone dose-response in a wide concentration range displays a bell-shaped curve, as already known for other hormones that typically interact with plasma membrane receptors (i.e. insulin, ANF etc.) and shows a maximum effect in the low physiological range. The lack of effect at higher concentrations could be considered the expression of a desensitization or down-regulation phenomenon, by which the cells protect themselves against high hormone levels (24, 43).

In order to understand the possible involvement of the classical nuclear receptor (ER) we used well known inhibitors: tamoxifen that is a partial ER antagonist (12) and ICI 182,780 that is a pure ER antagonist (44). 17β-Estradiol action in RASM cells was completely prevented by cell treatment with low concentrations of either of these inhibitors. The nongenomic effects of estradiol in some cases are prevented by the cell treatment with Tamoxifen or ICI 182,780 (45, 46) in some cases are not (47, 48). In these cell lines the expression of both α and β ERs isoforms have been reported, although their absolute level in the vascular tissue is not yet well characterized (42). The group of Nadal has reported nongenomic estrogen actions in the endocrine pancreas that are not prevented by ICI 182,780, ascribing the estradiol effects to a new γ-adrenergic receptor (6). Our data suggest that the involved receptors, a putative membrane receptor, that following the Mannheim classification of nongenomic steroid actions may be called a classical estrogen receptor, is similar in

structure to or at least shares some analogy with, the nuclear receptor (8). For sake of simplicity we have here considered the receptor involved in the nongenomic response to be a plasma membrane receptor, in analogy with the situation found for other hormones. However, it should be remembered that this receptor may just as well be located in the cell interior; it could even be a nuclear receptor operating through a mechanism different from transcription factor activation (8).

As to the transduction mechanism, it might be useful to recall at this point that the Na^+/H^+ exchanger is activated either by kinase phosphorylation, by modulation of intracellular [Ca^{2+}] or both (19). Our data show that the activation of the Na^+/H^+ exchanger by estrogens is mediated at least by two parallel pathways and that their relative importance depends on the hormone concentration: The IP_3 and PKC pathway is functioning mainly at 10^{-10} M, as assessed by the use of inhibitors of protein kinase C and by immunoblot experiments of translocation of PKC from the cytosol to the membrane. The activation of PKC-α and the increase of both levels and production of IP_3 within one minute has been reported for the nongenomic effects of estrogens in different cells (33, 49). At 10^{-12} M there appears to be a major involvement of the MAPK pathway, confirmed by the use of inhibitors of MAPK pathway, and through immunoblotting experiments. The activation of these pathways was prevented by cell treatment with ICI 182,780.

The participation of the MAPK pathway has recently been reported in a wide variety of genomic and nongenomic effects of estrogens (50-55), even though the physiological response appears to be very much dependent on the cellular context. In addition, recent results suggest that thyroid hormones and estrogens, despite their different structures and biological effects, show similarities in the mechanism of their nongenomic actions, even though the physiological significance of this coincidence is not known yet (54-56).

The relationship between pH and vascular tone has been known since the paper of Gaskell, and over a century later the molecular mechanisms of the pH effects on vascular smooth muscle are still poorly understood (57). Intracellular pH affects the degree of contraction and relaxation of the smooth muscle and, in general, a direct relationship exists between alkalinization and muscle contraction. Intracellular acidification decreases the affinity of calcium ions for the myofibrils, thus decreasing the vascular tone. On the other hand, following alkalinization, the increased affinity of calcium ions for the myofibrils leads to an increased vascular tone. Changes of pH can alter the force development in smooth muscle affecting the rate of myosin ATPase activity, the sensitivity of the contractile units to Ca^{2+}, the fluxes of Ca^{2+} into the cell, the transmitter release, the competition at Ca^{2+}-binding sites etc. (18). In an attempt to understand the physiological meaning of the increase in pH_i in a low range of hormone concentrations, we examined the effects of two α-adrenergic agonists on RASM cells, norepinephrine and phenylephrine. Our data show that these two vasoconstrictors caused an increase in intracellular pH superimposable to that of estrogen, with an identical time-course, and with no potentiation of the effects when the 17β-estradiol was given together with the α-adrenergic agonists.

It has been reported that the activity of the nuclear estrogen receptor may be stimulated also in the absence of an estrogen ligand (58). Multiple signaling pathways initiated by growth factors (59) or hormones (60) stimulate nuclear receptor activities. We suggest that the observed effects of estradiol on intracellular pH of RASM cells represent another example of the reported cross-talk between different steroid receptors as well as between different signaling pathways (6).

A simplified scheme for the smooth muscle relaxation indicates that estrogen can modify the expression of adrenergic receptors that in turn could give rise to an increase in cAMP, PKA activation, and myosin light chain kinase (MLCK) phosphorylation with

consequent muscle relaxation (15). In general, an increase in cAMP and activation of PKA in mammalian cells inhibits the activity of the Na^+/H^+ exchanger (61). RASM cells after a short-term treatment with estrogen did not show any increase in intracellular cAMP; this could be a factor that at the low estrogen concentrations allows the increase of intracellular pH and muscle contraction.

In conclusion the increase of intracellular pH due to 17β-estradiol is the consequence of three different factors: a) the activation of PKC, b) the increase of $[Ca^{2+}]_i$ due to IP_3, c) the activation of the MAPK pathway.

There are many potential targets for PKC in vascular smooth muscle, that could play a role in the modulation of the contractile process. First, PKC can phosphorylate the myosin light chain on sites different from those of MLCK, contributing to contraction (62). Second, PKC can phosphorylate other proteins such as calponin and caldesmon, that may depress the interaction between actin and phosphorylated myosin. In particular, caldesmon can be phosphorylated also by MAP kinase pathway and PKC can activate an early step of this kinase cascade (62). On the other hand the role of IP_3 in the release of intracellular calcium and in smooth muscle contraction is well known. The role of MAP kinase in contractile smooth muscle is not well understood, even though in the last few years there has been much interest on MAP kinase in this field (63). A relationship between MAPK pathway and smooth muscle contraction has been reported, since the specific inhibitor of MAPK kinase PD 98059 significantly inhibited the contraction to phenylephrine (64). A similar result has recently been obtained for ovine uterine artery (65).

We hypothesize that the increase of intracellular pH in response to low concentrations of estrogens may give a contribution, to the development of hypertension and atherosclerosis. A paper from one of us has shown that an increase of intracellular pH in human vascular smooth muscle cells can trigger cell proliferation in response to mitogens and affect cell

locomotion, key events in atherogenesis (26). All together these data suggest that the lower estrogen concentrations found in postmenopausal women might remove the hormone protective effects typical of premenopausal women, that, among other factors, regulate the pace of the Na^+/H^+ exchanger activity.

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Legends to Figures

Fig.1 Dose-response of 17β-estradiol on the proliferation of rat aortic smooth muscle cells. Results are reported as number of cells (%) with respect to control cells without hormone treatment and are the mean ± SD of at least 4-6 different experiments. The antiproliferative effect was significant in the physiological concentration range (10^{-10}-10^{-8} M). * $p< 0.01$ as from a Student's *t* test with respect to control.

Fig.2 Effect of 17β-estradiol on the steady-state intracellular pH in rat aortic smooth muscle cells. Upper panel: Dose-response of hormone effect on intracellular pH at steady state. Results are given as ΔpH_i/40 min over basal value and are the mean ± SD of at least 10 different experiments. * $p< 0.01$ starting from 10^{-10} M, with respect to baseline pH, as from a *t* test. Lower panel: Time-course of the effect of 17β-estradiol (10^{-12} M), on the intracellular pH. The effect of the inhibitor EIPA (10 μM) is shown. EIPA by itself did not affect the fluorescent signal. The graphs are representative of at least 10 similar experiments. The arrow indicates the addition of hormone, with or without EIPA

Fig.3 Effect of nuclear estrogen receptor inhibitors and of the stereoisomer 17α-estradiol on the intracellular pH at the steady state. Tamoxifen (10^{-7} M) and ICI 182,780 (10^{-8} M) were given to the cells together with 17β-estradiol (10^{-12} M) and none of them affected the fluorescent signal. The effect of the stereoisomer 17α-estradiol (10^{-12} M) alone is also reported. Results are shown as ΔpH_i/40 min over basal value and are mean ± SD of at least 3-10 different experiments.

Fig.4 Time-course of the effect of norepinephrine and phenylephrine on the steady state intracellular pH. Norepinephrine (Upper panel; NE) and phenylephrine (Lower panel; PHE) were tested at 10 μM. The effect of the inhibitor EIPA (10 μM) is also reported in the presence of each agonist. Results are representative of at least 6-10 similar experiments. The arrow indicates the addition of hormone/agonist, with or without EIPA

Fig. 5 Effect of inhibitors of tyrosine kinase, phospholipase C, protein kinase C and the MAPK pathway on the increase of intracellular pH by 17β-estradiol. The inhibitor concentration was 10^{-4} M for neomycin and H-7, and 10^{-7} M for U 0126, U 73122 and genistein; light-activated calphostin C was 1 μM, PD 98059 was 10 μM. All inhibitors except calphostin C were given to the cells 5 min before hormone addition whereas the samples containing calphostin C were light-activated with 30-min 'dark' and 30 min 'light' preincubation, before hormone treatment. Results are reported as ΔpH_i/40 min over basal value and are mean ± SD of at least three different experiments.

Fig. 6 Effect of inhibitors of protein kinase C and the MAPK pathway on the increase of intracellular pH by phenylephrine. Panel A: The protein kinase C inhibitor H-7 (100 μM) was given to the cells together with the hormone, whereas calphostin C (1 μM) was light-activated (see the Legend to Fig. 5). Panel B: Inhibitors of the MAPK pathway PD 98059 (10 μM) and U 0126 (100 nM) were given to the cells 5 min before hormone treatment and at the concentrations employed did not affect the fluorescent signal. Results are reported as ΔpH_i/40 min over basal value and are mean ± SD of at least 6-7 different experiments.

Fig. 7 Effect of 17β-estradiol on inositol 1,4,5 trisphosphate (IP_3) level and production. Panel A: Dose response of 17β-estradiol in a wide concentration range on IP_3 levels. Results are

reported as pmol/mg protein and are the mean ± SD of three different experiments. *P<0.05, at least, as from a Student's *t* test with respect to control. Panel B: Time-course of IP_3 production in rat aortic smooth muscle cells with (■) or without (○) 17β-estradiol (10^{-10} M). Results are reported as fmol/mg protein. Panel C: Effect of ICI 182,780 (1 μM) and U 73122 (10^{-7} M) on IP_3 production. Results are reported as fmol/mg protein and are the mean ± SD of 4-6 different experiments. P<0.001 as from a Student's *t* test with respect to control (*) or with respect to 17β-estradiol alone (°).

Fig. 8 Effect of the Ca^{2+} chelator BAPTA (25 μM) on the responses to 17β-estradiol (10^{-12} M) and phenylephrine (10^{-5} M) of the intracellular pH at the steady state. Results are the mean ± SD of 7-8 different experiments and are reported as ΔpH_i/40 min. BAPTA/AM at the concentration used did not affect the fluorescent signal.

Fig. 9 Effect of 17β-estradiol on PKC-α translocation. Panel A: Dose-response of 17β-estradiol (E) on the PKC-α translocation from cytosol to the membrane. Panel B: Effect of the nuclear estrogen receptor inhibitor ICI 182,780 (10^{-8} M) and of the phospholipase C inhibitor U 73122 (1 μM) on the translocation of PKC-α from cytosol (empty bars) to the membrane (filled bars) treated with and without 17β-estradiol (10^{-10} M). Data reported on the right side of both panels show the translocation of PKC-α determined by densitometric scanning and are the mean ± SD of three different experiments of Western Blotting. P<0.001 as from a *t* test with respect to its own control (C, *) or with respect to estrogen-treated cells (E, °). The β-actin was used to verify iso-loading of the samples.

Fig. 10 Effect of 17β-estradiol on the phosphorylation and content of the ERK2. Panel A : Dose-response of 17β-estradiol (E) on the levels and phosphorylated ERK2 (ERK2-P). Panel

B: Effects of the nuclear estrogen inhibitor receptor ICI 182,780 (10^{-8} M), the PKC inhibitor RO 31-8220 (1 μM), and the inhibitors of ERK phosphorylation U 0126 (100 nM) in RASM cells treated 15 min with 17β-estradiol (10^{-12} M). The right side of both panels shows the densitometric scanning and are the mean ± SD of at least three different experiments of Western blotting. $P<0.001$ as from a *t* test with respect to its own control (C, *) or with respect to estrogen-treated RASM cells (E, °). The β-actin was used to verify the iso-loading of the samples.

Fig. 11 Dose-response of 17β-estradiol on cAMP levels. The cAMP levels was measured after 15 min incubation with the hormone. Results are given as pmol/mg protein and are the mean ± SD of 4-6 different experiments.

Fig 1

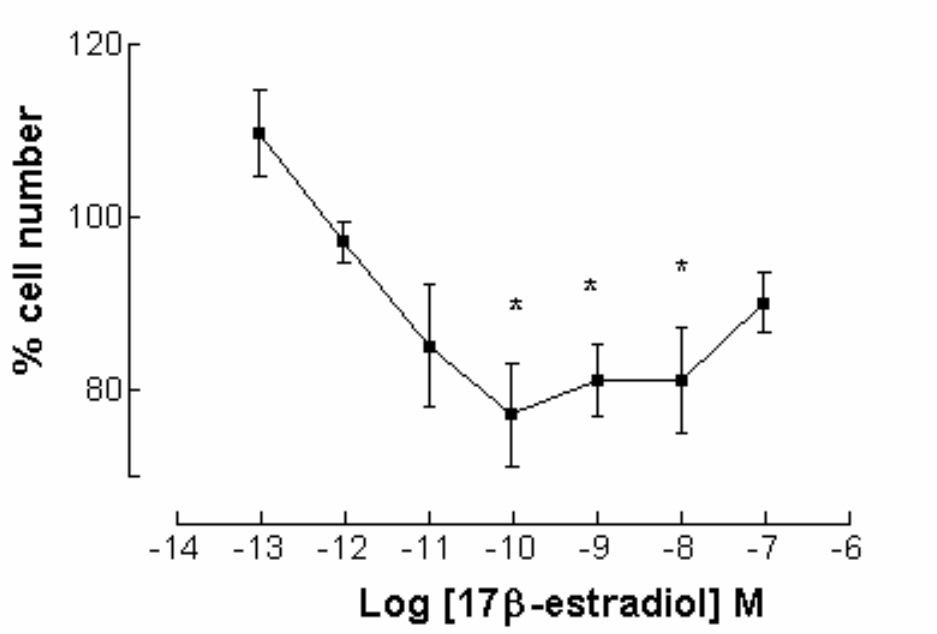
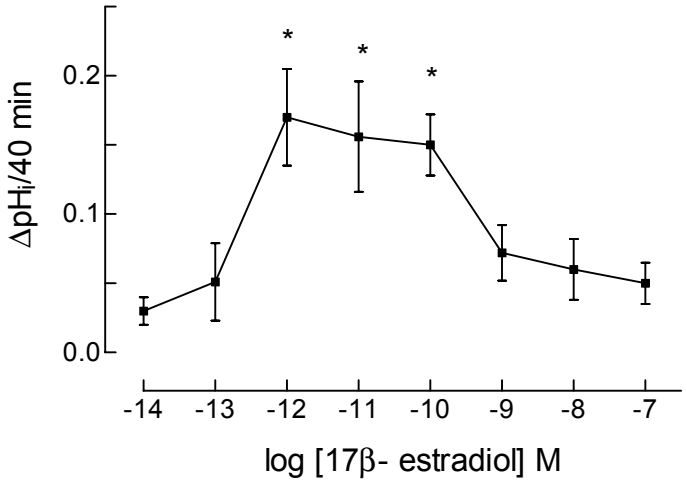


Fig 2


*
*
*
0.2
0.1
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ΔpHi/40 min
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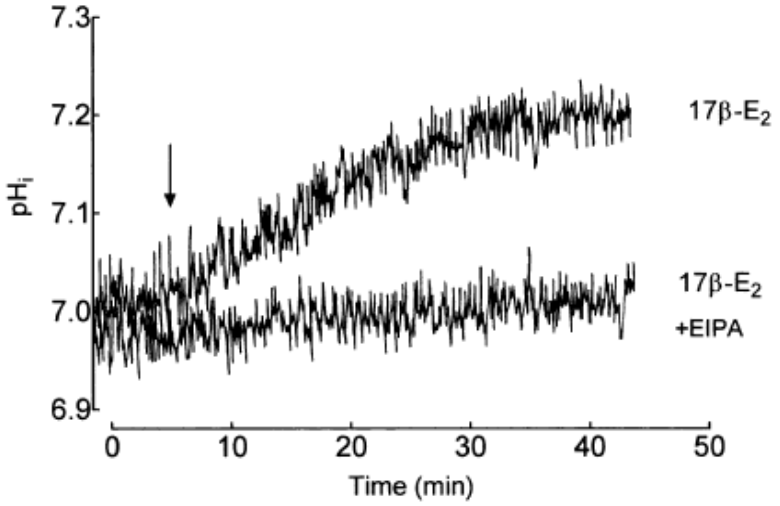
-13
-12
-11
-10
-9
-8
-7
log [17β- estradiol] M


7.3
7.2
7.1
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pHi
17β-E2
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+EIPA
0
10
20
30
40
50
Time (min)

Fig 3

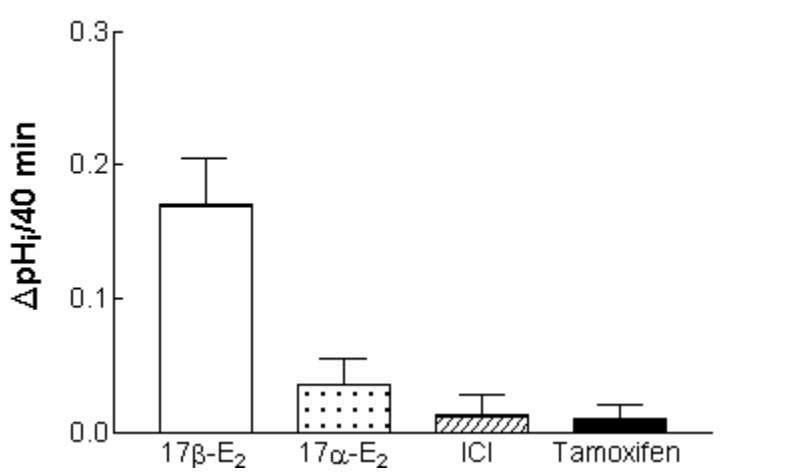
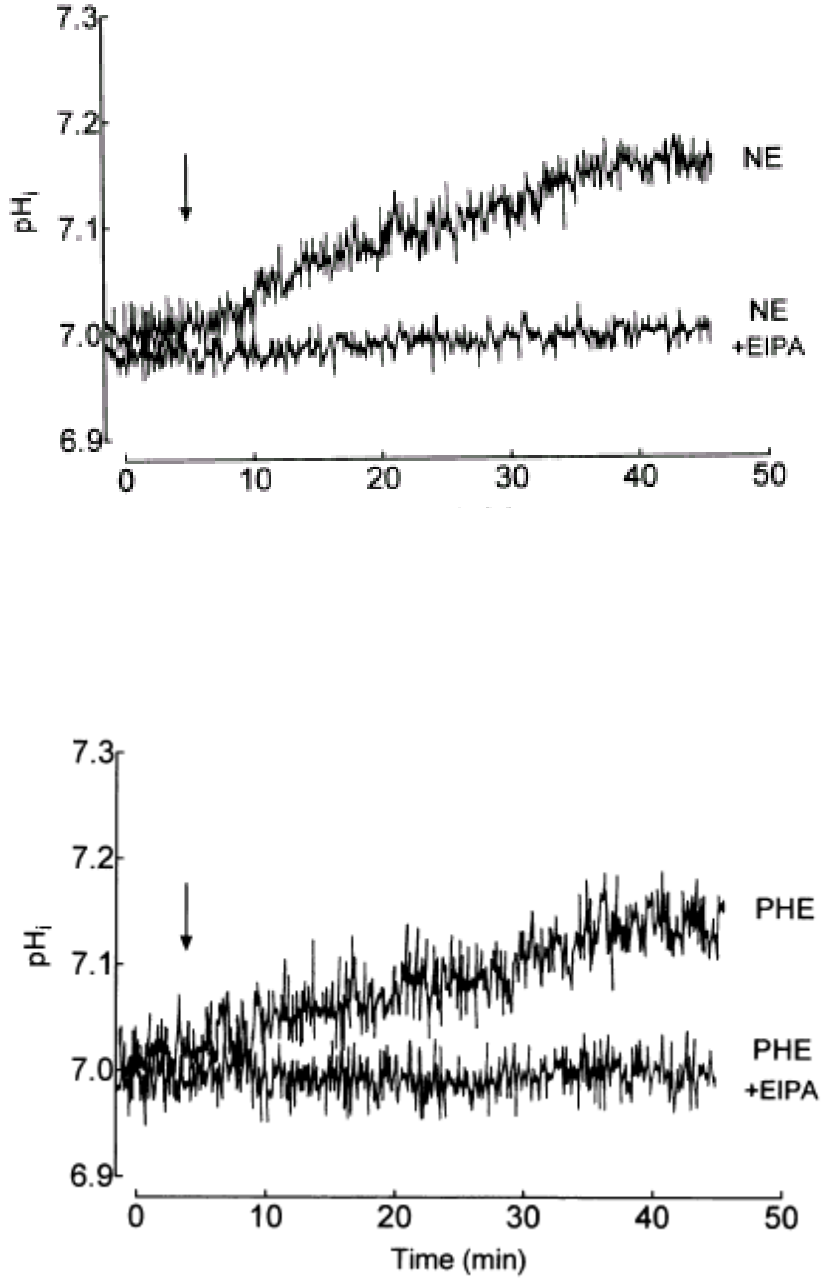


Fig 4



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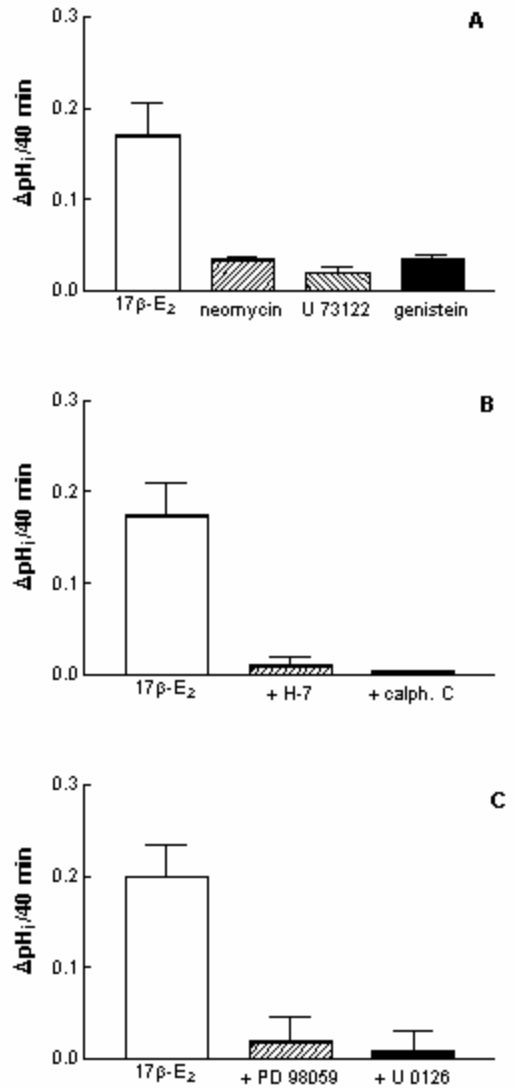
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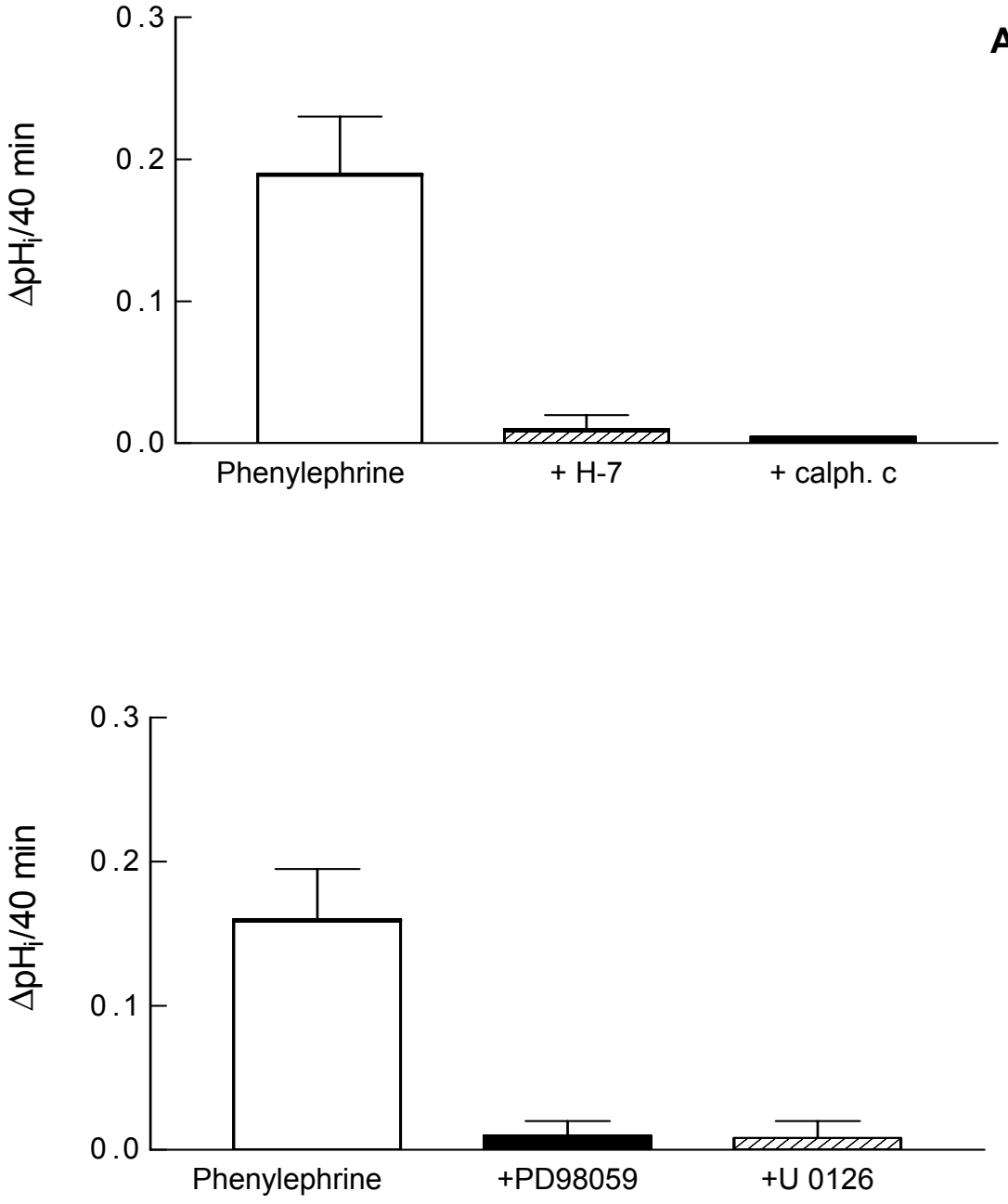
ΔpHi/40 min
0.3
0.2
0.1
0.0
17β-E2
neomycin
U 73122
genistein
B
ΔpHi/40 min
0.3
0.2
0.1
0.0
17β-E2
+ H-7
+ calph. C
C
ΔpHi/40 min
0.3
0.2
0.1
0.0
17β-E2
+ PD 98059
+ U 0126

Fig 6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0.2
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ΔpHi/40 min
Phenylephrine
+ H-7
+ calph. c
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ΔpHi/40 min
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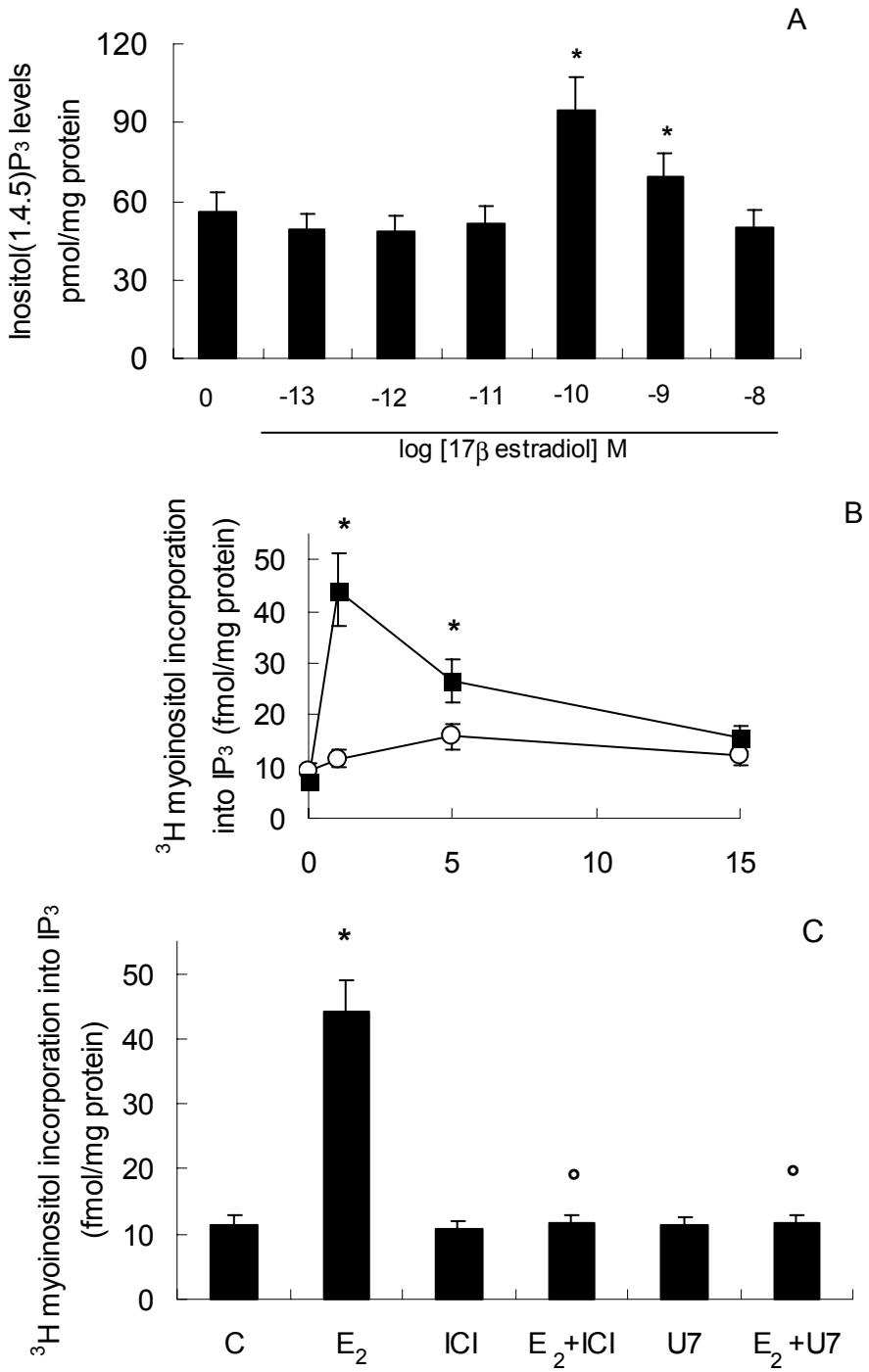
+PD98059
+U 0126

Fig 7


A
Inositol(1.4.5)P3 levels
pmol/mg protein
120
90
60
30
0
*
*
0
-13
-12
-11
-10
-9
-8
log [17β estradiol] M
B
3H myoinositol incorporation
into IP3 (fmol/mg protein)
50
40
30
20
10
0
*
*
0
5
10
15
C
3H myoinositol incorporation into IP3
(fmol/mg protein)
50
40
30
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0
*
°
°
C
E2
ICI
E2+ICI
U7
E2 +U7

Fig 8

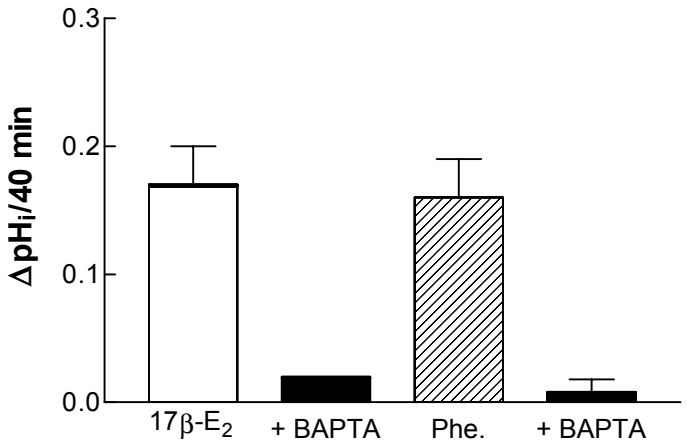
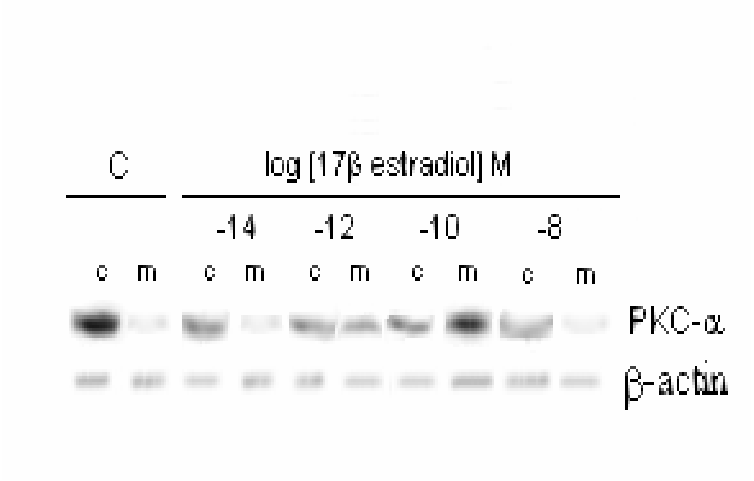


Fig 9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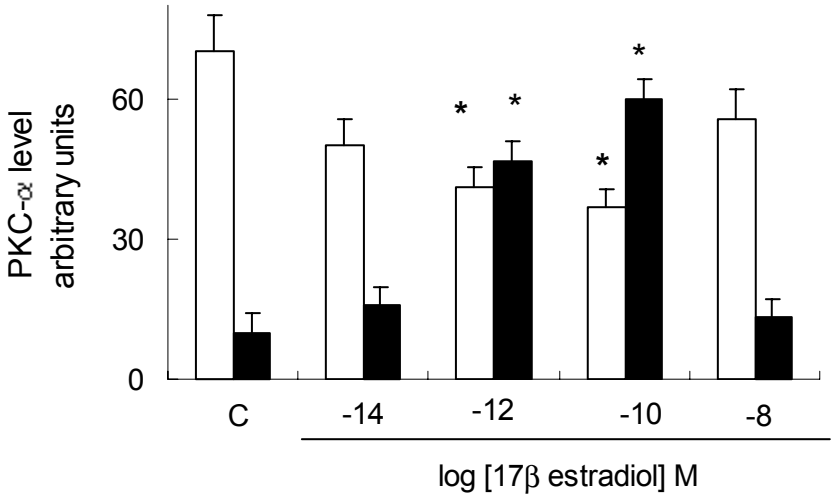
-12
-10
-8
c m c m c m c m c m
PKC-α
β-actin

A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C
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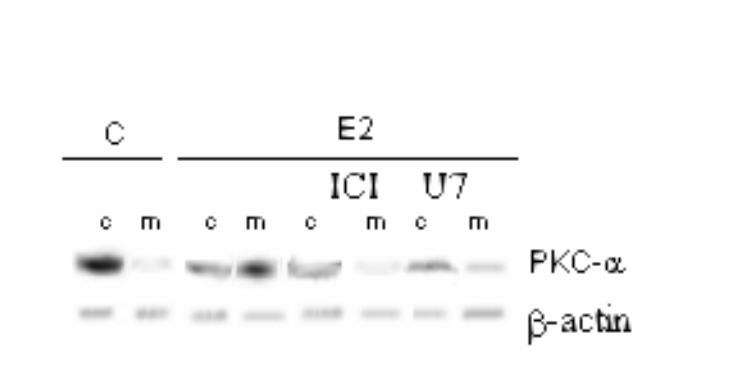
-12
-10
-8
log [17β estradiol] M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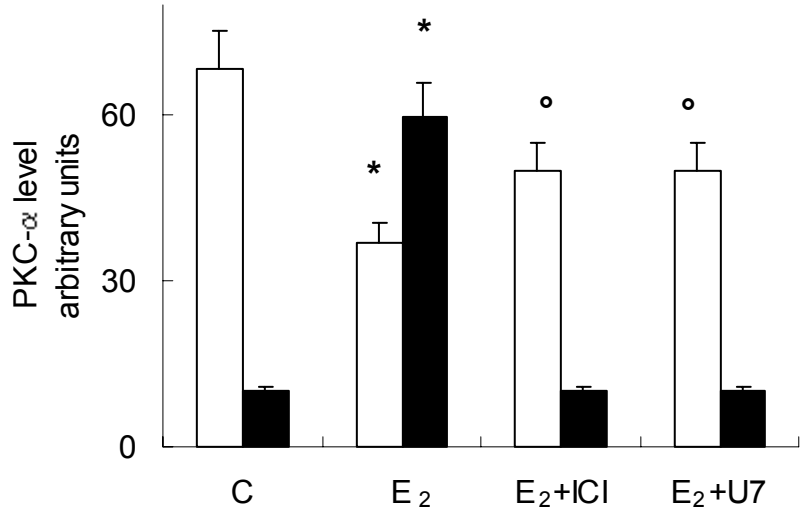
c m c m c m c m c m
PKC-α
β-actin

B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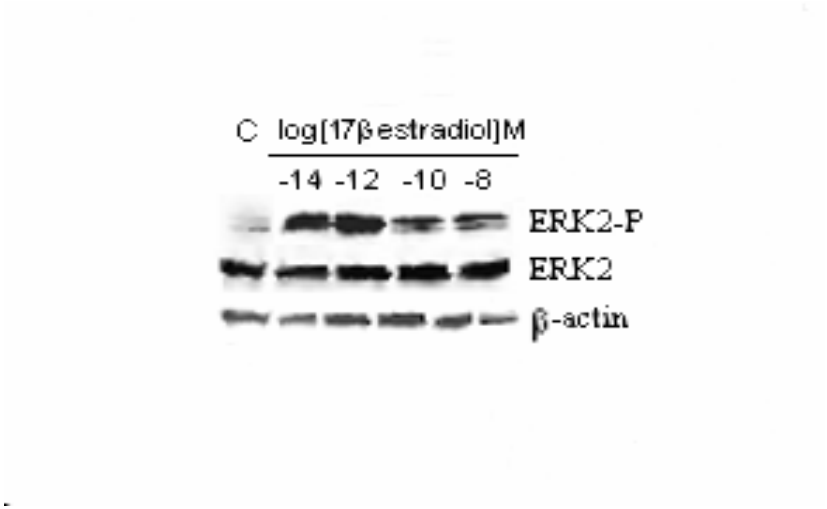
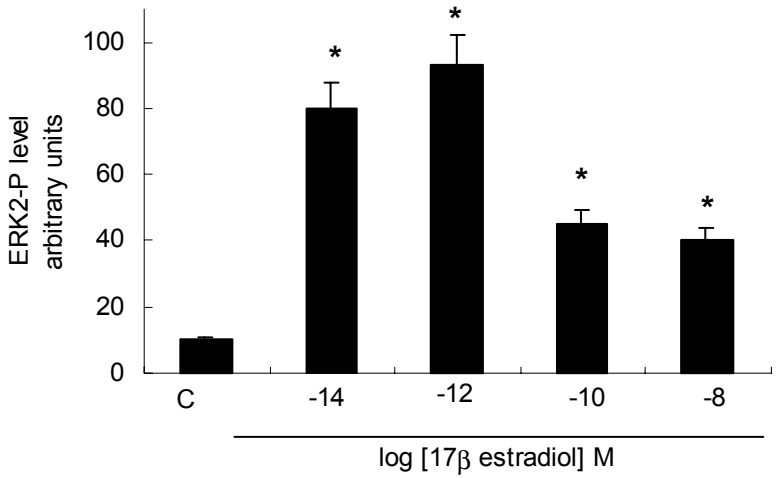
arbitrary units
60
30
0
C
E 2
E2+ICI
E2+U7

Fig 10

A


C log[17βestradiol]M
-14 -12 -10 -8
ERK2-P
ERK2
β-actin


ERK2-P level
arbitrary units
100
80
60
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*
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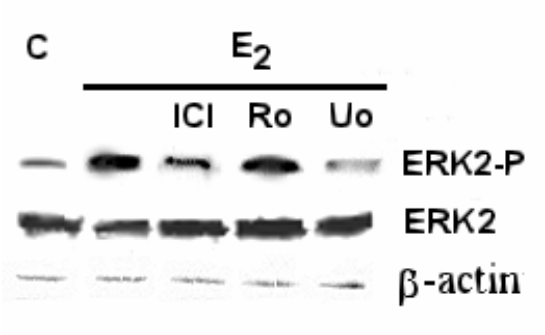
*
*
C
-14
-12
-10
-8
log [17β estradiol] M

B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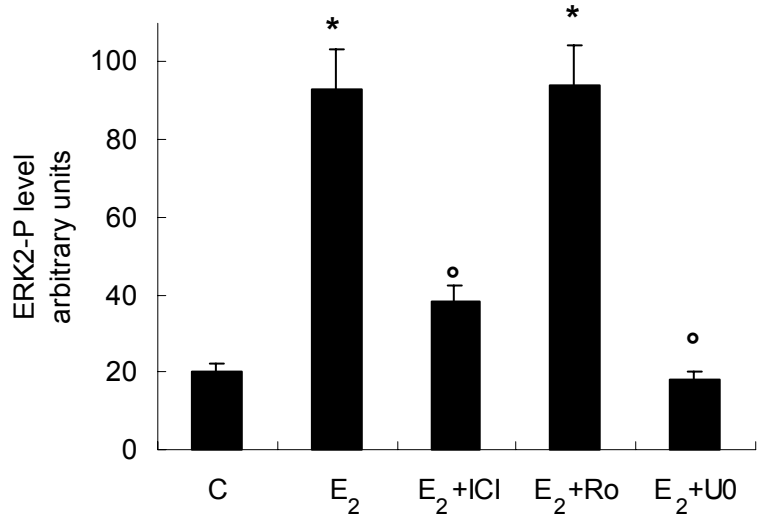
ICI Ro Uo
ERK2-P
ERK2
β-actin


ERK2-P level
arbitrary units
100
80
60
40
20
0
*
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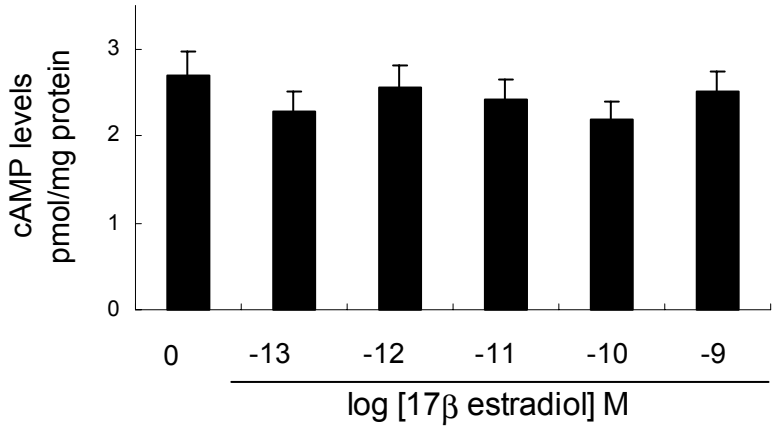
°
°
C
E2
E2+ICI
E2+Ro
E2+U0

Fig 11


cAMP levels
pmol/mg protein
3
2
1
0
0
-13
-12
-11
-10
-9
log [17β estradiol] M