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Estradiol Signaling via Sequestrable Surface Receptors

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ABSTRACT

Estradiol (E_2)-signaling is widely considered to be exclusively mediated through the transcription-regulating intracellular estrogen receptor (ER) α and ERβ. The aim of this study was to investigate transcription-independent E_2-signaling in mouse IC-21 macrophages. E_2 and E_2-BSA induce a rapid rise in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) of Fura-2 loaded IC-21 cells as examined by spectrofluorometry. These changes in $[Ca^{2+}]_i$ can be inhibited by pertussis toxin, but not by the ER-blockers tamoxifen and raloxifene. The E_2-signaling initiated at the plasma membrane is mediated through neither ERα nor ERβ, but rather through a novel G protein-coupled membrane E_2-receptor as revealed by RT-PCR, flow cytometry, and confocal laser scanning microscopy. A special feature of this E_2-receptor is its sequestration upon agonist stimulation. Sequestration depends on energy and temperature, and it proceeds through a clathrin- and caveolin-independent pathway. (*Endocrinology* **142:** 1669–1677, 2001)

ESTROGENS exert a broad spectrum of activities on a wide variety of cells and tissues and are also known to promote cancer of the mammary gland and endometrium. According to the current view, estrogens mediate their activities through transcription-regulating intracellular estrogen receptors (iER). These proteins contain several domains for estrogen binding, nuclear localization, dimerization, DNA-binding, and transactivation that impart iERs the ability to activate or repress specific estrogen-responsive genes (1–5). For a long time, it has been accepted that there exists only one type of receptor, now termed iERα. Recent findings, however, have revealed the existence of still another intracellular receptor, the so-called iERβ (6–10).

There is also increasing evidence for transcription-independent actions of estrogens, as for other steroid hormones too (11). These actions manifest themselves as rapid responses of target cells in the range of seconds to minutes. For instance, E_2 can induce a fast rise in the intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) due to influx of external Ca^{2+} and/or release of Ca^{2+} from intracellular Ca^{2+} stores (12–17). Such nongenomic actions are initiated at the plasma membrane and are postulated to be mediated by plasma membrane-associated estrogen receptors (mER). The current debate focuses on the nature and properties of these mERs. There is some evidence that the mER is identical with at least one form of the iER. For instance, Pappas *et al.* have shown in pituitary cells that the mER is very similar—if not identical—with iER, because mER cross-reacts with iER-recognizing antibodies (18). In accordance, recent transfection studies with iERα and iERβ complementary DNAs in CHO cells have revealed about 3% of both iERα and iERβ in plasma membrane enriched fractions (19). However, there are also reports stating that the mER is different to iERα and iERβ (13, 20–22). Here, we show the existence of mER in the murine macrophage cell line IC-21 with totally different properties as hitherto revealed: the mER is neither ERα nor ERβ, but is a G protein-coupled receptor which mediates both Ca^{2+} mobilization and Ca^{2+} influx, and which is sequestrable upon agonist stimulation.

Materials and Methods

Cell culture

Cells of the mouse macrophage cell line IC-21 were obtained from the American Type Culture Collection (ATCC-No. TIB-186; Manassas, VA) and were grown in IMDM medium/L-glutamine (Life Technologies, Inc., Eggenstein, Germany) supplemented with 10% FCS, 50 μM β-mercaptoethanol and 3.024 g $NaHCO_3$ at 37 C, 5% CO_2 and 96% humidity. They were subcultured once per week for maximally eight passages and incubated in serum-free medium for 24 h before experimentation.

Chemicals

17β-estradiol (E_2), 17β-estradiol 6-(*O*-carboxymethyl)oxime/BSA (E_2-BSA), 17α-estradiol, tamoxifen, nifedipine, verapamil, and pertussis toxin were from Sigma (St. Quentin, Fallavier, France), and raloxifene from Eli Lilly & Co. (Saint-Cloud, France). 1-(6-((17β-3-metoxyestra-1,3,5(10)-trien-17-yl)-amino)hexyl)-1H-pyrrole-2,5-dione (U-73122) and 1-(6-((17β-3metoxyestra-1,3,5(10)-trien-17-yl)-amino)-hexyl)-2,5-pyrrolidine-2,5-dione (U-73343) were from BIOMOL Research Laboratory (Plymouth, MA). Fura-2/AM was from Amersham Pharmacia Biotech (Les Ulis, France). 17β-estradiol 6-(O-carboxymethyl)oxime: BSA-fluoresceine isothiocyanate conjugate (E_2-BSA-FITC) was from Sigma (Deisenhofen, Germany) and Concanavalin A (Con A)-rhodamine from Vector (Burlingame, CA). Vectashield was delivered from Vector (Burlingame, CA) and 1,4-diazobicyclo-[2.2.2]octane (DABCO) from Merck & Co., Inc. (Darmstadt, Germany).

Ca^{2+} measurement

IC-21 cells were assayed for $[Ca^{2+}]_i$ as described (23). In brief, cells were grown on poly-L-lysine-coated glass coverslips until confluence and then loaded with 1 μM Fura-2/AM for 30 min at room temperature. The Ca^{2+} was measured in a temperature-controlled (37 C) Hitachi

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F-2000 spectrofluorometer. Steroids and reagents were added directly to the cuvette under continuous stirring (13, 15). Estrogens were dissolved in ethanol; the final concentration of ethanol never exceeded 0.01%, and this concentration had no effect on $[Ca^{2+}]_i$. E_2-BSA was treated with charcoal to remove any free E_2 or 17β-estradiol 6-(O-carboxymethyl-)oxime (24). Charcoal treatment had no effect on the ability of E_2-BSA to increase $[Ca^{2+}]_i$ (13). The Fura-2 fluorescence response to $[Ca^{2+}]_i$ was calibrated from the ratio of the 340/380 nm fluorescence values after subtraction of the background fluorescence of the cells at 340 nm and 380 nm (25). The dissociation constant of Fura-2-Ca^{2+} complex was taken as 224 nM. The values for R_{max} and R_{min} were calculated from measurements made using 25 μM digitonin, and 4 mM EGTA and enough Tris base to raise the pH to 8.3 or higher. Each measurement on Fura-2 loaded cells was followed by a parallel experiment under identical conditions with cells not loaded with Fura-2.

Labeling with E_2-BSA-FITC

IC-21 cells were washed twice with phosphate-buffered salt solution (PBS^+; 140 mM NaCl, 2.7 mM KCl, 6.4 mM Na_2HPO_4, 1.4 mM KH_2PO_4, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, pH 7.2), and incubated at the indicated temperatures for varying periods with 1.5×10^{-5} M E_2-BSA-FITC, or with BSA-FITC or BSA alone as controls. For internalization experiments, intact IC-21 cells were incubated at room temperature or 37 C for 15 min or 1 h with E_2-BSA-FITC, BSA-FITC or Con A-rhodamine (1:50) or a rat antimouse F4/80 antibody (2 μg/ml; gift from H. Mossmann, MPI for Immunobiology, Freiburg, Germany) and with Biotin-SP-conjugated AffiniPure mouse antirat IgG (H+L) (1:500; Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) as a secondary antibody and streptavidin-fluoresceine (6 $\mu g/10^7$ cells; Amersham Pharmacia Biotech, Braunschweig, Germany). Colocalization was performed in intact cells using LysoTracker Red DND-99 (10 μM; Molecular Probes, Inc., Göttingen, Germany) or transferrin conjugated with tetramethylrhodamine (20 μg/ml; Molecular Probes, Inc., Göttingen, Germany). Then, the samples were postfixed with 1% paraformaldehyde (PFA) (26). Cells prefixed with 0.5% PFA were incubated with the anticlathrin antibody HC (N-19) (2 μg/ml; Santa Cruz Biotechnology, Inc., Heidelberg, Germany) and with a donkey antigoat-Cy3 antibody (1:200; gift from P. Traub, MPI for Cell Biology, Ladenburg, Germany) as secondary antibody, or with the anti-caveolin antibody caveolin-1 (N-20) (2 μg/ml; Santa Cruz Biotechnology, Inc.) using a TRITC-conjugated AffiniPure goat antirabbit IgG (H+L) antibody (1:80; Jackson ImmunoResearch Laboratories, Inc.) as secondary antibody. The cells were postfixed with 3% PFA (23).

Localization of ER

Intact IC-21 cells as well as cells prefixed with 0.5% PFA and permeabilized with PBS^+ containing 0.05% Tween-20 and 0.5% BSA were labeled with the different ER-antibodies ERα (MC-20), ERα (H-184), and ERβ (Y-19) (all Santa Cruz Biotechnology, Inc.) in concentrations of 2 μg/ml for 1 h at room temperature. Antirabbit IgG (whole molecule) FITC conjugate (working dilution 1:320; Sigma, Deisenhofen, Germany) and a donkey antigoat-FITC antibody (working dilution 1:100; gift from P. Traub, MPI for Cell Biology, Ladenburg, Germany) were used as secondary antibodies for 45 min. The cells were postfixed with 1% PFA (23, 26).

Confocal laser scanning microscopy

IC-21 cells (2×10^6 cells/ml) were allowed to adhere onto poly-L-lysine-coated glass coverslips overnight, then labeled as described above, and embedded in a 1:1 (vol/vol) mixture of glycerol and vectashield containing 2% (wt/vol) DABCO (23). The confocal laser scanning microscope (CLSM) Leica Corp. TCS NT version 1.5.451 (Leica Corp. Lasertechnik, Heidelberg, Germany) was used for analysis of the specimens with FITC fluorescence excitation at 488 nm or Cy3 and TRITC fluorescence at 568 nm, respectively. Z-series optical sections taken at 0.5 μm intervals were evaluated using Adobe Photoshop 5.0 for Windows and Corel-Draw 8 for Windows (15, 27).

Flow cytometry

Aliquots of 150 μl IC-21 cells (10^7 cells/ml in PBS^+) were centrifuged, and the cell pellets were labeled as described above. Cells were analyzed in a FACScan (Becton Dickinson and Co., Sunnyvale, CA) with a sample size of 10,000 cells gated on the basis of forward and side scatter. The data were stored and processed using the FACScan software (26).

RNA isolation

RNA was isolated from IC-21 cells and ovaries removed from 8- to 10-week-old C57BL/10 mice according to the GTC/CsCl method (28).

RT-PCR

The initial random-primed RT was performed with 1 μg of total RNA, M-MLV Reverse Transcriptase (Promega Corp., Madison), dNTPs (PCR Nucleotid Mix; Roche Molecular Biochemicals, Mannheim, Germany), and random primer (Perkin-Elmer Corp., Weiterstadt, Germany) in a MJ Minicycler (MJ Research, Inc., Biozym, Hess. Oldendorf, Germany) for 10 min at 25 C, 1 h at 42 C and 5 min at 95 C. Thereafter, the samples were purified with a QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany). For PCR, we used the template complementary DNA, *Taq* DNA Polymerase (Promega Corp., Madison, WI), dNTPs (PCR Nucleotid Mix; Roche Molecular Biochemicals), and six different oligonucleotide primer pairs. The carboxy terminus of the ERα was probed with two different primer pairs: (1) ERαP2–1434 (5′-ACAGGAATCAAGGTAAATGTGTGG-3′) and ERαM1–1807 (5′-CTCCAGGAGCAGGTCATAGAGG-3′); as well as (2) ERαP9–1350 (5′-GGCTGGAGATTCTGATGAFTGG-3′) and ERαM5–1935 (5′-GGGTATGTAGTAGGTTTGTAAGG-3′). The primer pair (3) ERαP16–589 (5′-CTACTACCTGGAGAACGAGCC-3′) and ERαM21–1029 (5′-GAAGCACCCATTTCATTTCGGC-3′) was used for the DNA-binding domain of ERα. The DNA-binding domain of ERβ was probed with the primer pair (4) ERβP5–224 (5′-CTTGCCTGTAAACAGAGAGACC-3′) and ERβM4–709 (5′-GACGGCTCACTAGCACATTGG-3′). The steroid binding domain of the ERβ was probed with the primer pairs (5) ERβP7–710 (5′-CAATGTGCTAGTGAGCCGTCC-3′) and ERβM4–1209 (5′-CTGCTGCTGGGAAGAGATTCC-3′) and (6) ERβP3–855 (5′-CAAGTCCGCCTCTTGGAAAGC-3′) and ERβM1–1160 (5′-CATCTGTCACTGCGTTCAATAGG-3′). The amplification was performed with 36 cycles at 94 C for 1 min, at 56 C for 1 min, and at 72 C for 1 min and at the end of the last cycle for 15 min at 72 C.

DNA sequencing

PCR fragments were separated in 2% Tris borate-EGTA gels, eluted, and cloned into the vector pGEM-TEasy (Promega Corp., Madison). The clones were sequenced with Thermo Sequenase fluorescent-labeled sequencing kit (Amersham Pharmacia Biotech), and analyzed with the LICOR sequencer (MWG Biotech, Ebersberg, Germany).

Results

E_2-induced rise in $[Ca^{2+}]_i$

At the physiological concentration of 1 nM, 17β-estradiol (E_2) induced a rapid increase in $[Ca^{2+}]_i$ by about 90–150 nM within 5 sec (Fig. 1A). This Ca^{2+} rise dropped after 20–40 sec and, then, turned into a sustained plateau. At 0.1 nM, E_2 caused a weaker Ca^{2+} spike by only about 50 nM Ca^{2+} (Fig. 1A). In contrast, 17α-estradiol did not induce any significant increase in $[Ca^{2+}]_i$ (Fig. 1A). Moreover, a rise in $[Ca^{2+}]_i$ could also be induced by 100 nM plasma membrane-impermeable E_2-BSA conjugate, whereas BSA alone did not influence the $[Ca^{2+}]_i$ (Fig. 1B). It is not clear why 100 times higher E_2-BSA concentrations were required to elicit the same response as free E_2. One reason may be that, because of steric hindrance, only one or two of the E_2 molecules bound to BSA are able to induce a Ca^{2+} response. Another reason may be that coupling of E_2 to BSA via carboxymethyl oxime (CMO) re-

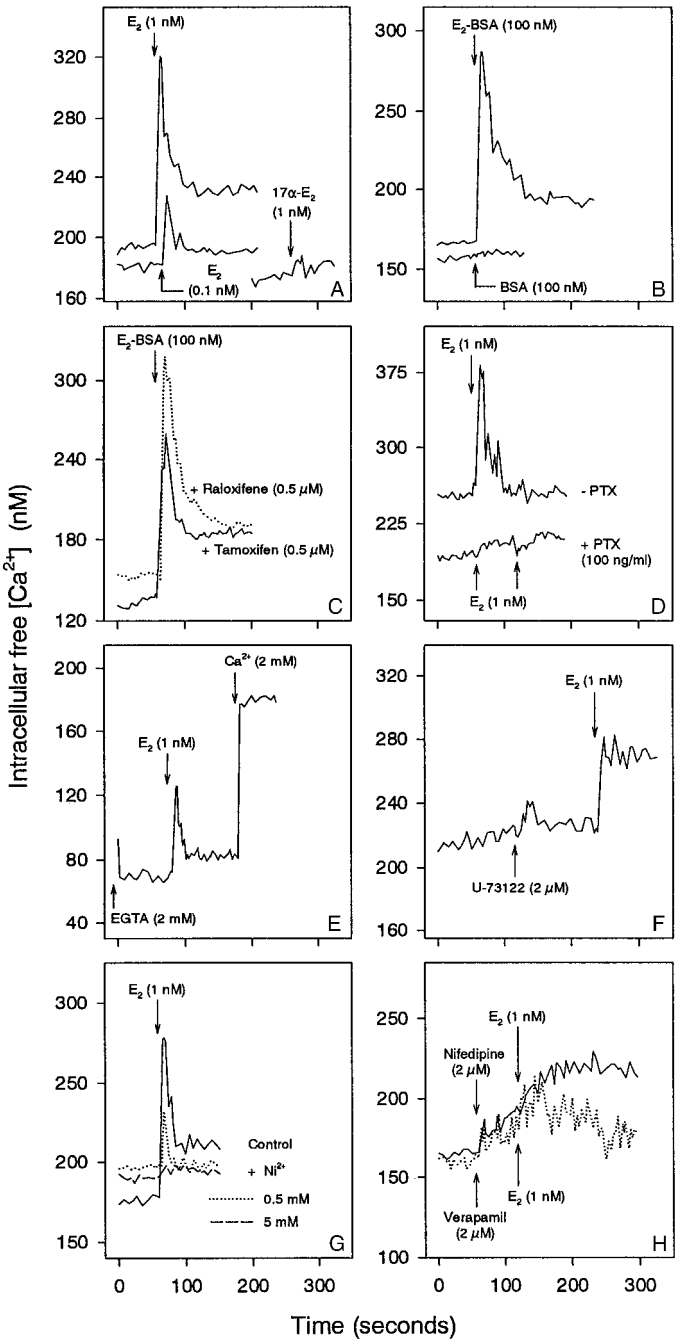

FIG. 1. Calcium responses of confluent IC-21 cells to estrogens. A, 17β-estradiol (E_2) but not 17α-estradiol (17α-E_2) induce a dose-dependent increase in $[Ca^{2+}]_i$. B, E_2 conjugated to BSA also induces a transient Ca^{2+} spike, but not BSA alone. C, Preincubation with raloxifene and tamoxifen for 4 h does not prevent the E_2-BSA-induced Ca^{2+} spike. D, Incubation of cells with 100 ng/ml pertussis toxin for 16 h (+ PTX) inhibits the E_2 effect on $[Ca^{2+}]_i$. E, The E_2-induced Ca^{2+} spike is lowered by removal of external Ca^{2+} with 2 mM EGTA. F, Preincubation with the direct phospholipase C inhibitor U-73122 for 2 min reduced the E_2-induced rise in $[Ca^{2+}]_i$ by about 50%. G, Pretreatment of cells with different concentrations of the Ca^{2+} channel blocker Ni^{2+} for 5 min gradually inhibited the E_2-induced Ca^{2+} spike. H, The two blockers of voltage-gated Ca^{2+} channels nifedipine and verapamil reduced the E_2-induced increase in $[Ca^{2+}]_i$. *Arrows* indicate addition of the indicated substances.

duces the capacity of E_2 to increase $[Ca^{2+}]_i$ (13). In this context, it is also noteworthy that we removed any E_2 and E_2-CMO possibly released from E_2-BSA with the stripped-charcoal technique described by Lieberherr *et al.* (13). Repeated additions of E_2 or E_2-BSA leads to repeated Ca^{2+} spikes (23). Even a pretreatment of IC-21 cells with 1 or 10 nM E_2 for 4 h did not reduce the Ca^{2+} response to E_2 (data not shown). Pretreatment of cells with tamoxifen or raloxifene, which are blockers of classical iERs, prevented neither the E_2-BSA-induced nor the E_2-induced increase in $[Ca^{2+}]_i$ (Fig. 1C). However, pertussis toxin totally inhibited the E_2-induced Ca^{2+} spike (Fig. 1D).

The E_2-induced rise in $[Ca^{2+}]_i$ may be due to influx of extracellular Ca^{2+} and/or release of Ca^{2+} from intracellular Ca^{2+} stores. When extracellular Ca^{2+} was first removed by EGTA, 1 nM E_2 induced a smaller Ca^{2+} spike by only about 40–60 nM, due to the release of Ca^{2+} from intracellular Ca^{2+} stores (Fig. 1E). When intracellular Ca^{2+} mobilization was inhibited by the direct phospholipase C inhibitor U-73122, there was still an E_2-induced increase in $[Ca^{2+}]_i$ due to influx of extracellular Ca^{2+} (Fig. 1F). Partial inhibition of Ca^{2+} increase was achieved with Ni^{2+} concentrations known to inhibit the release of Ca^{2+} from intracellular stores (Fig. 1G, 0.5 mM), whereas the influx was completely inhibited by higher Ni^{2+} concentrations that also block plasma membrane Ca^{2+} channels (Fig. 1G, 5 mM). Also, the E_2-induced Ca^{2+} influx could be reduced with the two blockers of voltage-gated Ca^{2+} channels nifedipine (L-type channels) and verapamil (Fig. 1H).

Sequestrable surface E_2-binding sites

To test the presence of putative surface E_2-receptors, the IC-21 cells were incubated with the ligand E_2-BSA-FITC conjugate. After labeling for 5 sec, flow cytometry detected a significant increase in fluorescence intensity compared with unlabeled control cells (Fig. 2A). However, the fluorescence intensity increased gradually with progressing labeling periods reaching a maximum after about 1 h (Fig. 2A). In parallel, the cells were investigated by confocal laser scanning microspopy (CLSM). After labeling for 5 sec and 1 min, the fluorescence was exclusively localized on the cell surface. After 5 min, however, weak punctate fluorescence emerged inside of the cells at their periphery, besides surface fluorescence. This punctate fluorescence increased in intensity after labeling for 1 h and was distributed throughout the whole cytoplasm (Fig. 2B).

The internalized punctate E_2-BSA-FITC was not contained in acidic vesicles (Fig. 3A). The latter were stained with LysoTracker Red DND-99 and did not colocalize with the green punctate fluorescence of E_2-BSA-FITC (Fig. 3A). Also, the sequestrated E_2-BSA-FITC colocalized neither with clathrin as detected by anti-clathrin antibodies (Fig. 3B) and by transferrin-tetramethylrhodamin, which is an indicator of sequestration through clathrin-coated vesicles (Fig. 3C), nor with caveolin as monitored by anticaveolin antibodies (Fig. 3D). The internalization process could be inhibited by treatment of cells with hypertonic medium (Fig. 3E) but not by the tubulin-blocker nocodazole and the microfilament-blocker cytochalasin B (data not shown).

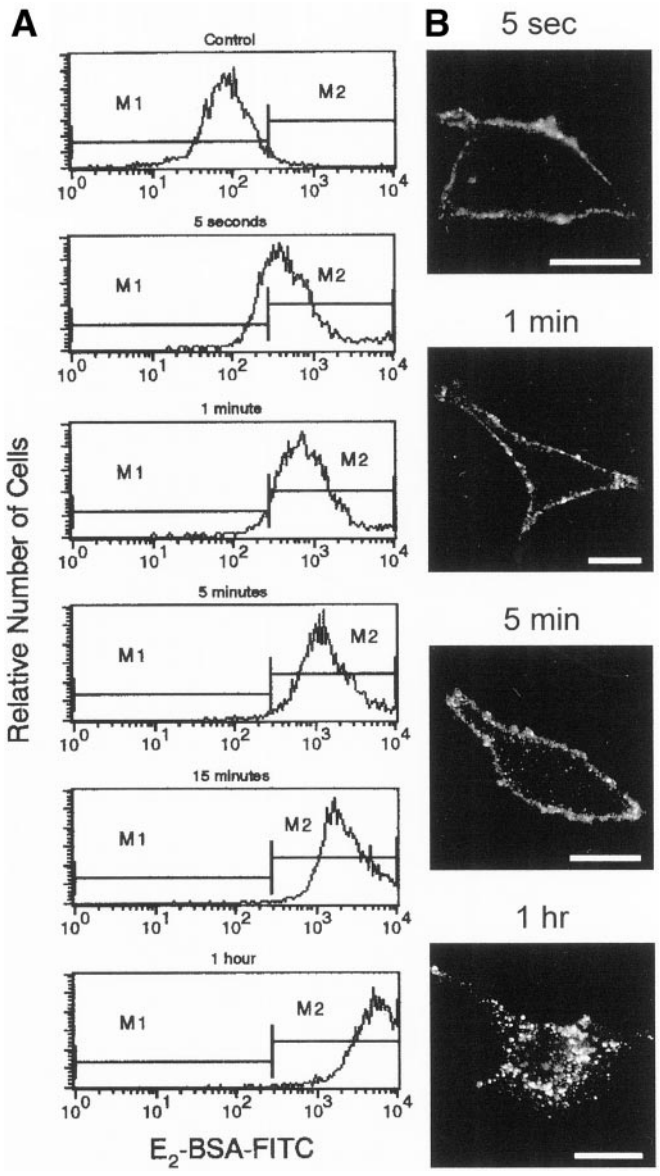

FIG. 2. Sequestration of surface bound E_2-BSA-FITC. Cells were labeled with E_2-BSA-FITC for varying periods, and fluorescence was recorded by flow cytometry (A) and CLSM (B). *Bars* represent 10 μm. M1, Area of unlabeled cells. M2, Area of E_2-BSA-FITC labeled cells.

Specificity of E_2-receptor sequestration

The internalization of surface E_2-receptors was selective. Incubation of cells with either BSA or BSA-FITC alone, or in combination with 1 nM E_2 for 15 min did not induce any sequestration, whereas control cells incubated with E_2-BSA-FITC revealed sequestrated fluorescence (Fig. 4, A and B). Moreover, internalization occurred neither with surface-bound ConA-rhodamine nor with the macrophage specific surface marker F4/80 identified by a rat monoclonal antibody against F4/80, even if the surface labelings were performed in the presence of E_2 (Fig. 4B).

The binding and the internalization of E_2-BSA-FITC were competitively reduced by 17β-E_2 and 17β-E_2-BSA but not by 17α-estradiol (Fig. 5A). By contrast, pretreatment of cells with 17β-E_2 for 2 h before incubation with E_2-BSA-FITC alone did not prevent sequestration (Fig. 3F). Neither testosterone nor testosterone-BSA nor 1-dehydrotestosterone were able to competitively reduce the sequestration of E_2-BSA-FITC (Fig. 5A). Pretreatment of IC-21 macrophages with pertussis toxin resulted in a decrease of internalized fluorescence intensity (Fig. 5A). However, the phospholipase C inhibitor U-73122 as well as its inactive control compound U-73343 did not block the internalization of surface-bound E_2-BSA-FITC (data not shown). Furthermore, the internalization of membrane E_2-receptors depended on energy. Depletion of ATP by sodium azide resulted in a decrease of the fluorescence intensity by about 30% (Fig. 5A), which was localized almost exclusively on the surface of IC-21 cells. Finally, the sequestration of membrane E_2-receptors was also dependent on temperature. Temperatures at 16 C and below largely inhibited the sequestration of surface-bound E_2-BSA-FITC. By contrast, the binding of E_2-BSA-FITC to the cell surface was not affected by temperature (Fig. 5B).

Intracellular ER

The presence of iERα and iERβ in IC-21 cells was first examined by RT-PCR. Using different primers of the carboxy terminus and the DNA-binding domain of ERα, RT-PCR revealed the expected bands of ERα in IC-21 cells and mouse uterus, which was used as a positive control (Fig. 6A). DNA sequencing confirmed that the PCR fragments derived from uterus and IC-21 cells contained the predicted regions of the ERα. However, RT-PCR did not detect any ERβ in IC-21 cells, whereas the uterus was ERβ positive (Fig. 6A). In accordance, incubation of IC-21 cells with the anti-ERβ antibody ERβ (Y-19) directed against an epitope corresponding to the amino terminus of the ERβ did not result in any significant labeling of intact or permeabilized IC-21 cells as detected by flow cytometry and CLSM (data not shown).

ERα was predominantly localized in the cytoplasm and to a lesser extent in the nucleus as detected in permeabilized cells by CLSM using the anti-ERα antibody ERα (MC-20) directed against an epitope in the carboxy terminus of the ERα as well as the anti-ERα antibody ERα (H-184) directed against an epitope in the amino terminus of the ERα (Fig. 6C). Both antibodies specifically reacted with permeabilized cells, because the antibody reaction could be competitively displaced by specific blocking peptides as revealed by flow cytometry (Fig. 6B). However, ERα was not accessible on the outer surface of intact cells as probed by flow cytometry (Fig. 6B) and CLSM (data not shown) using the two different ERα antibodies described above. Furthermore, no sequestration of peripheral ERα was found. After 1 h labeling with the anti-ERα antibodies in the presence of E_2, there was no increase in fluorescence intensity of intact cells. Moreover, when intact IC-21 cells were incubated with the ERα (MC-20) antibody and, in parallel, with E_2-BSA-FITC for 1 h, CLSM did reveal only internalized E_2-BSA-FITC, but not any internalized ERα (data not shown; see Fig. 2B).

Discussion

This study provides evidence for the existence of a transcription-independent E_2-signaling pathway in the mouse macrophage cell line IC-21. Indeed, E_2 at physiological con-

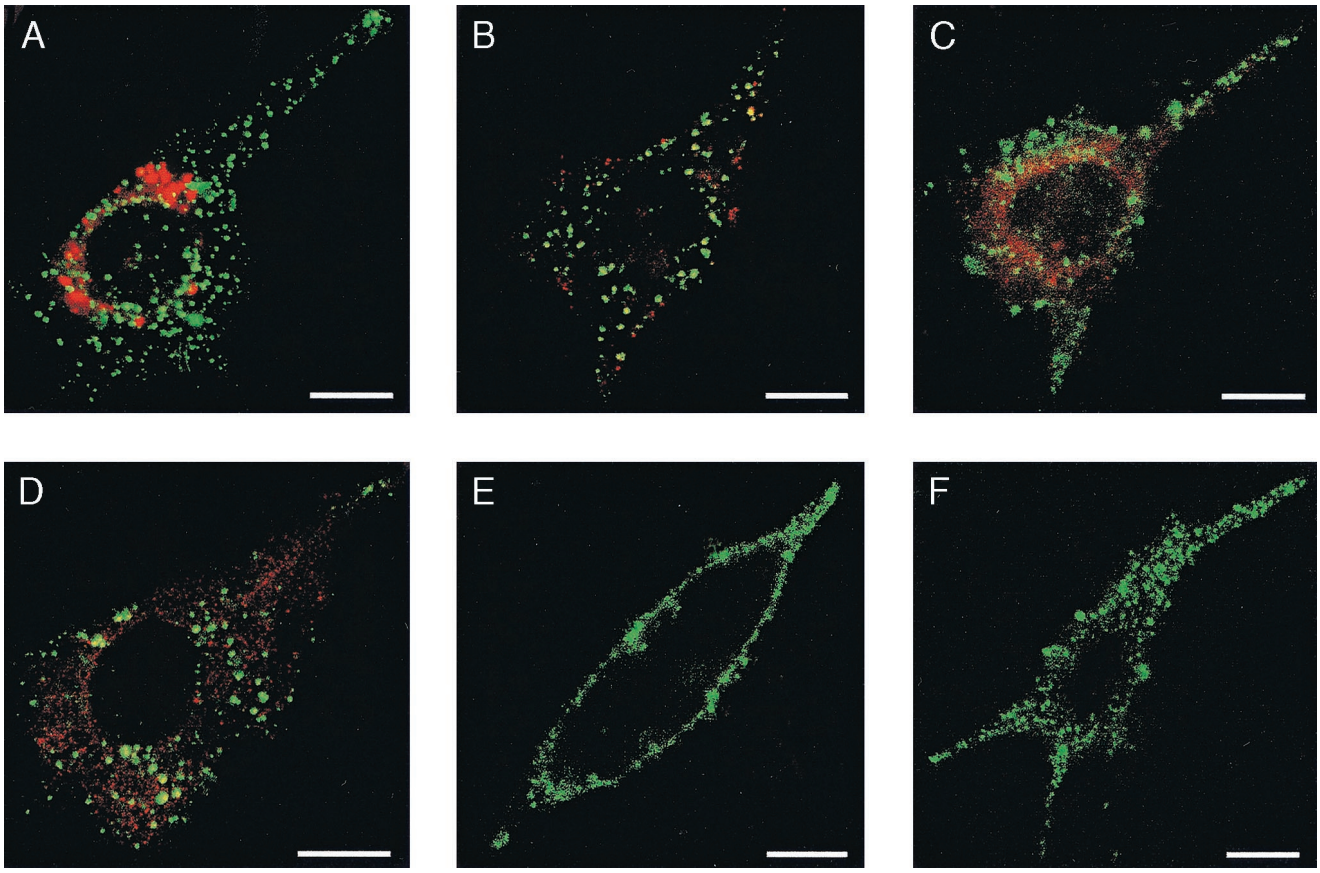

FIG. 3. CLSM colocalization of the sequestrated surface binding sites of E_2. A, Parallel labeling of IC-21 cells with E_2-BSA-FITC and LysoTracker Red DND-99, a marker of acidic vesicles, at 37 C for 1 h did not result in colocalization. B, Sequestration of E_2-BSA-FITC is independent of clathrin-coated vesicles as detected by an anti-clathrin antibody and the Cy3-labeled secondary antibody. C, Internalized E_2-BSA-FITC did not colocalize with internalized red transferrin-tetramethylrhodamine. D, No colocalization was observed after incubation of cells with E_2-BSA-FITC and an anti-caveolin antibody detected by TRITC-conjugated antibody. E, Pretreatment of cells with 0.45 M sucrose for 30 min inhibited the sequestration of E_2-BSA-FITC. F, Preincubation of IC-21 cells with 100 nM E_2 for 2 h did not prevent the internalization of E_2-BSA-FITC. *Bars* represent 10 μm.

centrations induces a rapid rise in $[Ca^{2+}]_i$, which is due to both influx of external Ca^{2+} and release of Ca^{2+} from intracellular Ca^{2+} stores. This is in accordance with previous results also showing E_2-induced Ca^{2+} rise due to both influx of extracellular Ca^{2+} and intracellular Ca^{2+} mobilization in rat osteoblasts (13), mouse T cells (15), and pig granulosa cells (29). However, there are also data showing only E_2-induced influx of Ca^{2+} in LNCaP human prostate cancer cells (14) and human spermatozoa (16) or only E_2-induced intracellular Ca^{2+} mobilization in chicken granulosa cells (12) and human peripheral monocytes (17). In IC-21 cells, the Ca^{2+} influx is not only a simple diffusion process but rather proceeds through Ca^{2+} channels that are completely blockable by Ni^{2+} and, in part, by nifedipine and verapamil. These data are in line with previous studies also showing that the E_2-induced rapid Ca^{2+} influx proceeds through Ca^{2+} channels, though the type of Ca^{2+} channels involved appears to depend on the cell type. For instance, there exist Ni^{2+}-sensitive Ca^{2+} channels in T cells (15) and pig granulosa cells (29), whereas osteoblasts contain predominantly voltage-gated Ca^{2+} channels (13).

The E_2-induced increase in $[Ca^{2+}]_i$ of IC-21 cells is initiated on the cell surface via specific E_2-receptors. This view is supported by our findings that also the plasma membrane-impermeable ligand E_2-BSA induces a rise in $[Ca^{2+}]_i$. In addition, the fluorescent conjugate E_2-BSA-FITC specifically binds to the surface of intact IC-21 cells as detected by flow cytometry and CLSM. Moreover, our data show that the rise in $[Ca^{2+}]_i$ can be blocked by pertussis toxin, and the phospholipase C inhibitor U-73122 inhibits the release of intracellular Ca^{2+}. Obviously, the surface E_2-receptors belong to that class of membrane receptors which are coupled to phospholipase C via a pertussis toxin-sensitive G protein. In accordance, recent studies show that E_2 activates $\beta\gamma$ subunits of a pertussis toxin-sensitive G protein coupled to a PLC-β2 in osteoblasts (21, 30).

The plasma membrane G protein-coupled receptors for E_2 (E_2-GPCR) in IC-21 cells exhibit properties that are typically found for other G protein-coupled receptors (GPCR). For instance, a wide variety of GPCRs, as *e.g.* the prototypic β_2-adrenergic receptor and the angiotensin II type 1A receptor, become sequestrated after ligand binding (31–33). Also, the E_2-GPCR become sequestrated a few minutes after binding of E_2 as visualized by labeling with E_2-BSA-FITC. This internalization process is ligand-specific, *i.e.* internalization of E_2-BSA-FITC is competitively inhibited by 17β-E_2 and

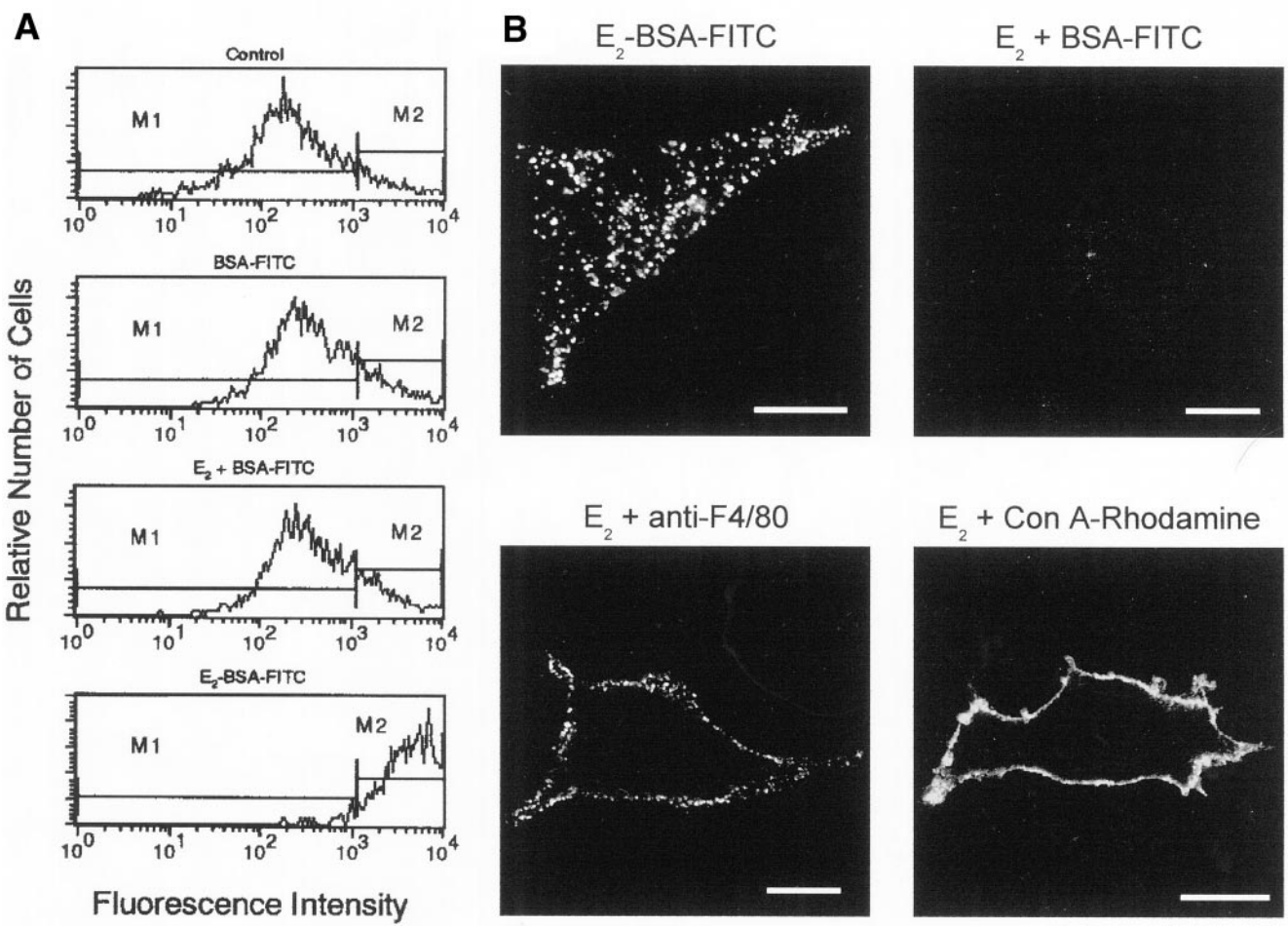

FIG. 4. Selective sequestration of E_2-surface binding sites. A, Flow cytometric analysis of IC-21 cells incubated with the indicated substances for 15 min. B, CLSM analysis of IC-21 cells incubated with the indicated substances for 15 min revealed internalization only in E_2-BSA-FITC-treated cells, but not in cells incubated with the other indicated substances. *Bars* represent 10 μm.

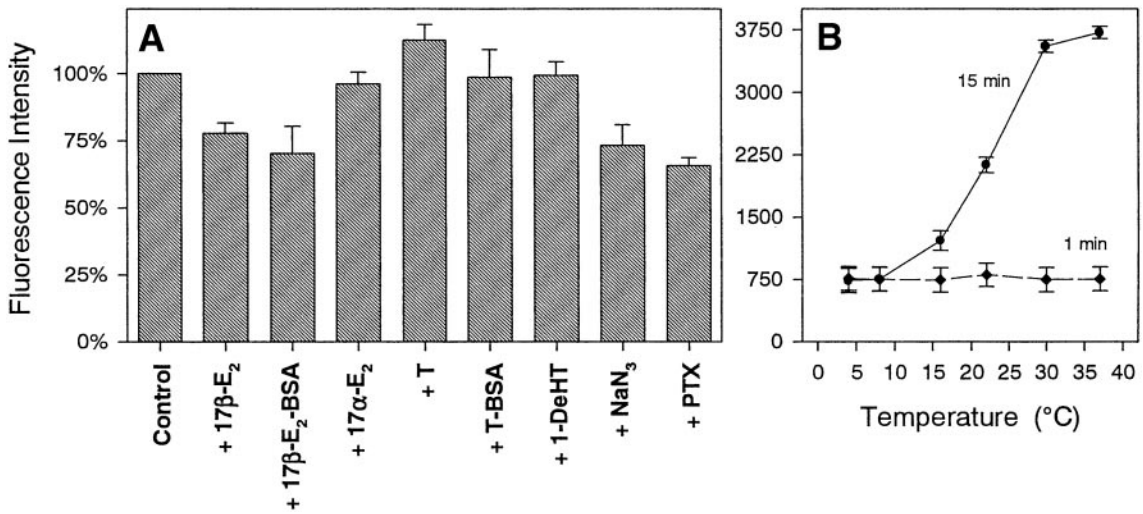

FIG. 5. Specificity of E_2-BSA-FITC sequestration. A, IC-21 cells were incubated for 15 min with E_2-BSA-FITC (10^{-6} M) in the absence (control) or in the presence of a 10-fold excess of different unlabeled hormones. Fluorescence intensity was analyzed by flow cytometry. Values normalized to controls are given as means ± SD from four different experiments. B, Cells were preincubated for 30 min at the indicated temperatures and then treated with 1.5×10^{-5} M E_2-BSA-FITC for 1 min or 15 min at the same temperatures. Values represent means ± SD from at least two different experiments.

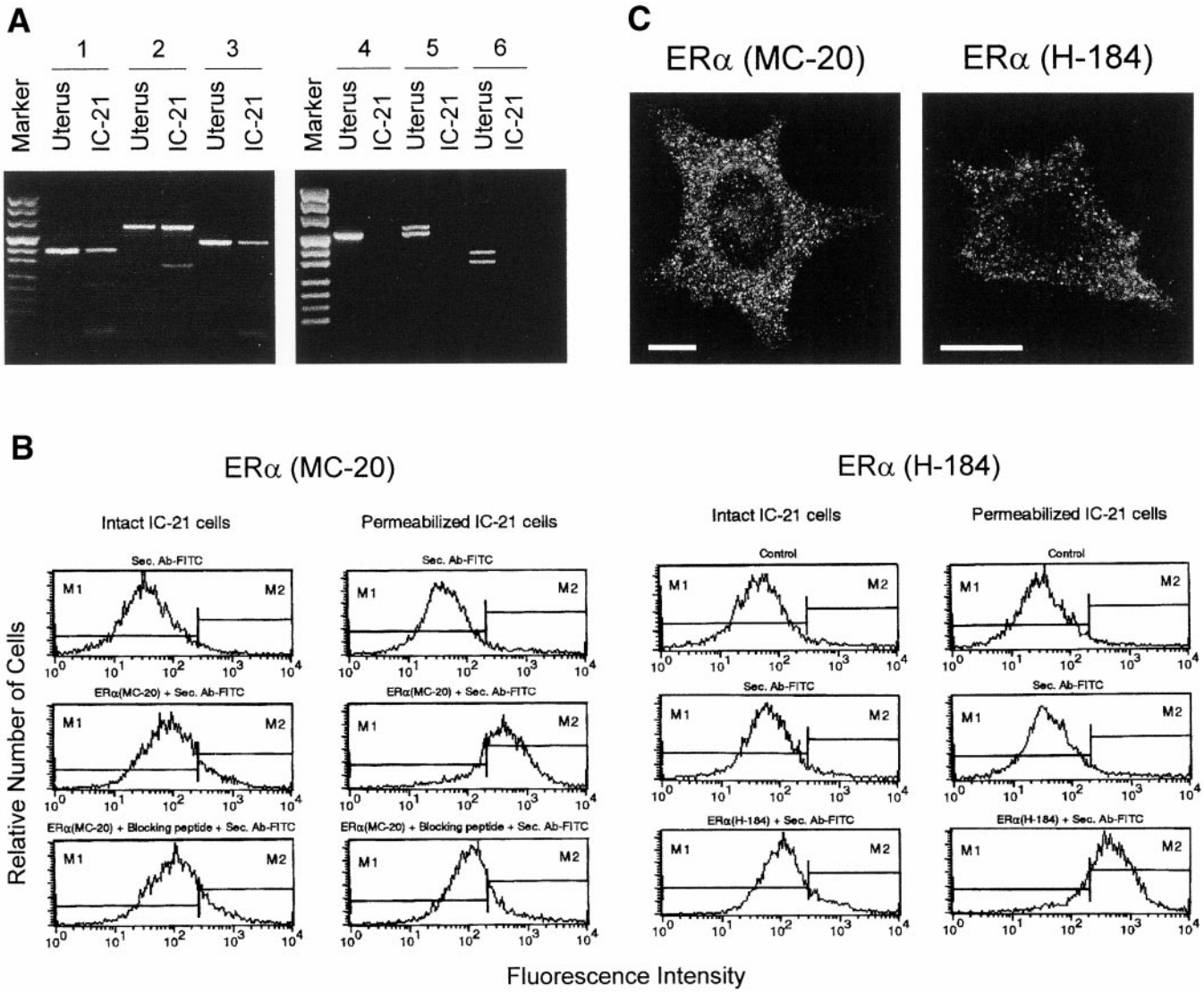

FIG. 6. Occurrence and localization of ERα and ERβ. A, RT-PCR with RNA isolated from mouse uterus and IC-21 cells, with markers (pUC mix, MBI Fermentas) on the *left*. The two primer pairs (1) ERαP2–1434/ERαM1–1807 and (2) ERαP9–1350/ERαM5–1935 spanned regions of the carboxy terminus of ERα. The primer pair (3) ERαP16–589/ERαM21–1029 was used for the DNA-binding domain of ERα. The DNA-binding domain of ERβ was probed with the primer pair (4) ERβP5–224/ERβM4–709 and the steroid binding domain of the ERβ with the primer pairs (5) ERβP7–710/ERβM4–1209 and (6) ERβP3–855/ERβM1–1160. B, Flow cytometry of intact and permeabilized IC-21 cells incubated for 1 h with two different ERα antibodies and their secondary FITC-labeled antibodies (Sec. Ab-FITC). The blocking peptide ERα (MC-20)P cannot competitively displace the slight increase in fluorescence of ERα (MC-20)-treated intact cells in contrast to permeabilized cells, where it totally blocks the strong fluorescence induced by ERα (MC-20) and its secondary fluorescent antibody. C, ERα is predominantly localized in the cytoplasm of permeabilized cells as revealed by CLSM using the anti-ERα antibodies ERα (MC-20) and ERα (H-184). *Bars* represent 10 μm.

17β-E_2-BSA, but not by 17α-estradiol and internalization depends on temperature and energy. Moreover, pertussis toxin reduces sequestration, indicating that E_2-GPCR internalization is dependent on Ca^{2+}. In addition, E_2-GPCR internalization is selective, *i.e.* only distinct plasma membrane domains are internalized, which exclude, for example, macrophage-specific surface molecules such as F4/80. In general, the sequestration of GPCR occurs via the clathrin-coated vesicle-mediated endocytotic pathway (34–36) or via caveolae (37, 38). The clathrin pathway can be prevented by hypertonic media (39–41). Also, we can find in IC-21 cells that the E_2-GPCR internalization is inhibited by hypertonic sucrose. Nevertheless, the E_2-GPCR sequestration is mediated by a clathrin- and caveolin-independent pathway because there is no colocalization of vesicles containing E_2-BSA-FITC with caveolin, clathrin, and transferrin. In accordance, there is some information available that internalization of GPCRs does not necessarily occur through clathrin- or caveolin-dependent pathways (35, 42). In general, GPCR sequestration is considered to be important for regulation of signaling, recycling, down-regulation and responsiveness or essential for the activation of specific signal transduction factors (33, 43–45). Though the reason for E_2-induced E_2-GPCR internalization is still unknown, a possible down-regulation does not seem very likely because pretreatment of cells with E_2 for 2 h did not prevent sequestration of

E_2-GPCRs and pretreatment for 4 h did not reduce the Ca^{2+} response to E_2. Thus, it seems more plausible that internalization of E_2-GPCR may be involved in the activation of specific signaling pathways.

Surface estrogen receptors, identical or structurally related to at least one form of the iER, have been recently localized in various cells such as GH_3/B6 rat pituitary tumor cells (18, 46), human monocytes (17), rabbit uterus cells (47), and transfected hamster ovary cells (19). By contrast, the surface E_2-GPCR of IC-21 cells are neither ERα nor ERβ. The latter is not expressed in IC-21 cells at all, and ERα is not accessible on the outer surface of intact cells, but can only be detected intracellularly, *i.e.* in the cytoplasm and to a lesser extent in the nucleus of permeabilized IC-21 cells. In accordance, other studies have also revealed that classical ERs can be localized in both the cytoplasm and the nucleus (48–51). On the basis of the cytoplasmic localization of ERα, it could be argued that a possible tight association of ERα with the cytoplasmic surface of the plasma membrane could lead to an activation of this ERα by E_2-BSA. However, this can be excluded because E_2-BSA conjugates bind to neither ERα nor ERβ as recently shown using several different assays (52). Moreover, ERα reveals properties which are clearly distinct from those of the E_2-GPCR on the surface of IC-21 macrophages. For instance, ERα cannot be induced to be internalized by E_2 or E_2-BSA-FITC, in contrast to the E_2-GPCR, though Kim *et al.* have demonstrated the occurrence of ERα in plasmalemmal caveolae (53). Moreover, the iER-blockers tamoxifen and raloxifene have no inhibitory effect on the rapid rise in $[Ca^{2+}]_i$ of IC-21 cells induced by both E_2 and E_2-BSA.

Recently, IC-21 cells have been also shown to contain sequestrable surface GPCRs for testosterone (T-GPCR) (23). However, T-GPCR exhibit properties different to those of E_2-GPCR. First, the E_2-GPCR mediates the E_2-induced increase in $[Ca^{2+}]_i$ via both Ca^{2+} release from intracellular stores and influx of extracellular Ca^{2+}, whereas testosterone induces, via T-GPCR, only a mobilization of Ca^{2+} from intracellular stores. The phospholipase C inhibitor U-73122 completely blocks the testosterone-induced raise in $[Ca^{2+}]_i$, whereas the E_2-induced raise is only reduced by approximately one half. Verapamil and nifedipine reduces the increase in $[Ca^{2+}]_i$ after E_2-treatment, whereas the testosterone-induced increase in $[Ca^{2+}]_i$ is unaffected by these drugs. Moreover, testosterone and testosterone-BSA are not able to compete with E_2 for the internalization of E_2-GPCR. Sequestration of T-GPCR, but not that of E_2-GPCR, is inhibited by the direct phospholipase C inhibitor U-73122 as well as by nocodazole and cytochalasin B. It remains to be seen as to whether the E_2-GPCR and the T-GPCR are two different receptors or there is only one receptor with different binding sites for E_2 and testosterone coupled to different signaling pathways.

Collectively, our data unequivocally show the presence of functional novel E_2-GPCR in plasma membranes of IC-21 cells that do not mediate the classical genomic ER-response, but rather initiate a transcription-independent E_2-signaling pathway involving Ca^{2+} as one of several other possible intracellular mediators.

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