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ARTICLE

Synthetic Estrogen Derivatives Demonstrate the Functionality of Intracellular GPR30

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ABSTRACT Estrogen mediates its effects through multiple cellular receptors. In addition to the classical nuclear estrogen receptors (ERα and ERβ), estrogen also signals through the seven-transmembrane G-protein-coupled receptor (GPCR) GPR30. Although estrogen is a cell-permeable ligand, it is often assumed that all GPCRs function solely as cell surface receptors. Our previous results showed that GPR30 appeared to be expressed predominantly in the endoplasmic reticulum. A critical question that arises is whether this localization represents the site of functional receptor. To address this question, we synthesized a collection of cell-permeable and cell-impermeable estrogen derivatives. We hypothesized that if functional GPR30 were expressed at the cell surface, both permeable and impermeable derivatives would show activity. However, if functional GPR30 were predominantly intracellular, like ERα, only the permeable ligands should show activity. Cell permeability was assessed using cells expressing ERα as a model intracellular estrogen-binding receptor. Our results reveal that despite exhibiting similar binding affinities for GPR30, only the cell-permeable ligands are capable of stimulating rapid calcium mobilization and phosphoinositide 3-kinase (PI3K) activation. We conclude that GPR30 expressed intracellularly is capable of initiating cellular signaling and that there is insufficient GPR30 expressed on the cell surface to initiate signaling in response to impermeable ligands in the cell lines examined. To our knowledge, this is the first definitive demonstration of a functional intracellular transmembrane estrogen receptor.

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Estrogen is a critical hormone in the development and homeostasis of many organs, particularly those involved in reproduction. It also plays an important role in the development and treatment of breast cancer due to the expression of the classical estrogen receptor, ERα, a member of the nuclear family of transcription factor receptors, in a high percentage of breast cancers, making it a target of therapeutic treatment with selective ER modulators (SERMs), such as tamoxifen, and more recently aromatase inhibitors. Estrogen is also involved in the development of osteoporosis, coronary heart disease, and other conditions (*1*).

GPR30 is a recently identified estrogen-responsive seven-transmembrane G-protein-coupled receptor (GPCR). It was originally cloned as an orphan GPCR by a number of groups in the late 1990s (*2–5*) and subsequently shown to be required for estrogen-mediated signaling in breast cancer cell lines (*6, 7*). Cell signaling through GPR30 occurs at least in part through G-protein-mediated transactivation of epidermal growth factor receptor (EGFR) (*6*), a mechanism employed by numerous GPCRs (*8*). Since its initial characterization, GPR30 has been shown to mediate the transcriptional regulation of numerous genes (*9–12*) and to be involved in the regulation of cell growth (*13–15*).

Recently, we (*16*) and others (*17*) demonstrated that GPR30 expression results in estrogen-binding sites localized to the site of GPR30 expression, consistent with the idea that GPR30 is an estrogen-binding receptor. Unexpectedly, our results indicated that GPR30 was primarily expressed in the endoplasmic reticulum with no detectable receptor expressed on the cell surface (*16*). This result was in contrast to the expected localization of a GPCR to the plasma membrane (*18*) and concurrent and subsequent reports describing cell surface localiza-

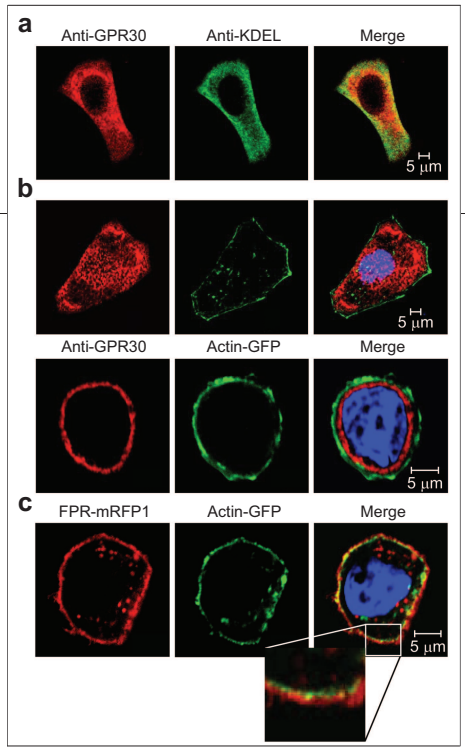

Figure 1. Localization of endogenously expressed GPR30 in SKBr3 breast cancer cells. SKBr3 cells were stained with anti-KDEL antibodies (a) or transfected with either pEGFP–actin alone (b) or pEGFP–actin and an FPR–mRFP1 fusion protein (c). For visualization of endogenous GPR30 in both a large, well-spread cell (a and b, upper panels) and a small, rounded cell (b, lower panels), cells were fixed, permeabilized, and stained with anti-GPR30 antibodies. For visualization of the FPR, a representative plasma membrane-localized GPCR, in a small, rounded cell (c), cells were fixed without subsequent treatment. Results are representative of three independent experiments.

tion of GPR30 (*17, 19, 20*). The existence of intracellular GPCRs is a topic of much current investigation and has been hypothesized for a number of GPCRs, particularly those with lipophilic ligands (*21, 22*). On the other hand, many GPCRs, such as odorant receptors, appear to express poorly at the cell surface and exhibit significant retention in the endoplasmic reticulum (*23*). Despite this controversy over the nature of intracellular GPR30 expression, the fundamental question remains as to whether an endogenous intracellular pool of GPR30 is capable of signaling or, alternatively, whether a small pool of GPR30 expressed on the cell surface is responsible for the receptor's signaling activity, as recently proposed (*20*). Although the majority of GPCR ligands are charged and therefore membrane-impermeable, estrogen is freely permeable to cellular membranes (*24*), making the former scenario a viable possibility. The high lipophilicity of the recently described GPR30-specific ligand G-1 suggests that this ligand is also cell-permeable (*25*).

Estradiol-conjugated bovine serum albumin (BSA) has been widely used as a membrane-impermeable form of estrogen to test for the presence of cell-surface ERs. However, the presence of noncovalently associated estradiol, the nature of the covalent linkage, and the molar ratio of estradiol to BSA (typically 30:1) raise questions regarding the suitability of its use (*26, 27*). To address the question of the functionality and localization of GPR30, we designed a series of 17β-estradiol (E_2) derivatives containing a 17α-phenylethynyl linker, which we have previously demonstrated permits binding to both classical ERs and GPR30 (*16*), allowing for the incorporation of either a neutral or charged group distal to the estrogen moiety. Our results demonstrate that activation of GPR30 by these derivatives correlates with the membrane permeability of the ligand. This suggests first of all that GPR30 expressed on the endoplasmic reticulum is active and second that there is no active receptor expressed at the cell surface in the cell lines we examined.

RESULTS AND DISCUSSION

We have previously reported that in cells transiently expressing GPR30 (as a GFP fusion), the receptor is expressed predominantly in the endoplasmic reticulum as assessed by colocalization with appropriate markers. Furthermore, a similar intracellular pattern of endogenous GPR30 expression was observed in a number of cancer cell lines (MCF-7, SKBr3, JEG, and Hec50co). To assess whether this pool of intracellular receptor was capable of binding ligand, we synthesized a fluorescent

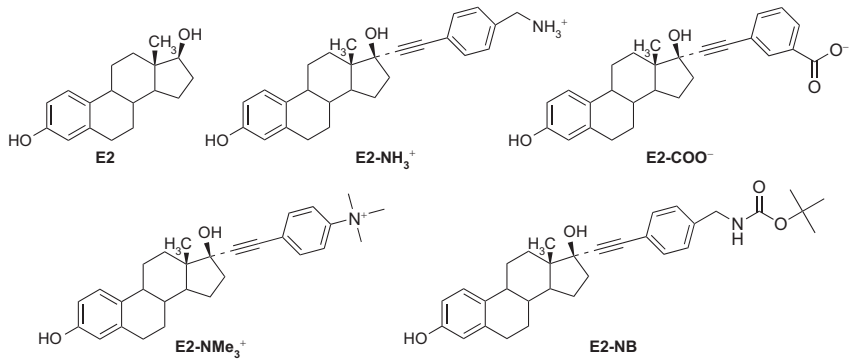

Figure 2. Structures of E2 and four novel estrogen derivatives. The estrogen derivatives are ionizable (E2-NH_3^+ and E2-COO^-), permanently charged (E2-NMe_3^+), or neutral (E2-NB).

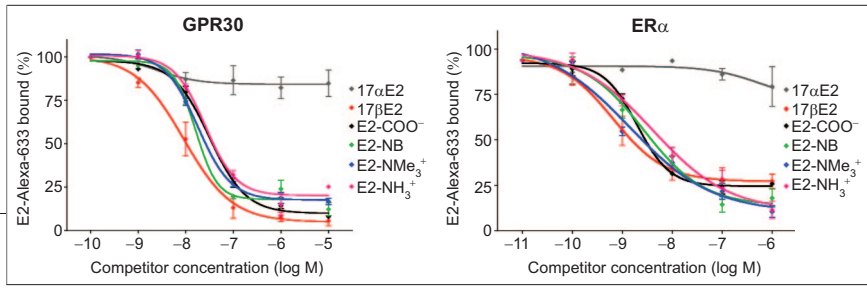

Figure 3. Binding of estrogen derivatives to GPR30 and ERα. Competition binding studies were carried out using COS-7 cells transfected with either GPR30–GFP or ERα–GFP and the fluorescent estrogen, E2-Alexa633. Data are means ± standard error of the mean (SEM) of at least three independent experiments fit to a sigmoidal dose response with Graphpad Prism. Resulting K_i values are shown in Table 1.

estrogen derivative, which demonstrated that the GPR30 localized in the endoplasmic reticulum is functional with respect to estrogen binding (*16*). Furthermore, binding of this highly charged (due to charges on the fluorophore) ligand to both ERs and GPR30 was only observed upon permeabilization of the cells, consistent with the idea that both receptor types are expressed intracellularly. Contrary to our observations however, others have reported finding GPR30 expressed on the cell surface (*17, 19, 20*).

To confirm that endogenously expressed GPR30 is also found in the endoplasmic reticulum, we compared GPR30 expression in SKBr3 breast cancer cells to markers of either the endoplasmic reticulum (anti-KDEL) or the actin cytoskeleton (actin–GFP). We observed that the staining pattern of GPR30 in these cells coincides with the endoplasmic reticulum marker (Figure 1, panel a) and is found internal to the submembranous actin cytoskeleton (Figure 1, panel b). The intracellular expression of GPR30 is observed in well-spread cells (Figure 1, panel b, upper panels), as well as the smaller rounded cells that make up 40–50% of cultured SKBr3 cells (Figure 1, panel b, lower panels). In the latter case, GPR30 staining appeared annular but staining of the nucleus reveals that GPR30 is expressed in a cytoplasmic region between the nucleus and the actin cytoskeleton. As a control, we expressed a carboxy-terminal red fluorescent protein (RFP) fusion of the *N*-formyl peptide receptor (FPR), a protein known to be expressed at the cell surface. Although the FPR overlapped with the actin cytoskeleton at some sites, it was clearly expressed external to the actin–GFP, consistent with the expected localization at the plasma membrane (Figure 1, panel c).

TABLE 1. Binding affinity measurements of estrogen derivatives

	Affinity (K_i, nM (95% confidence interval))	
Compound	**ERα**	**GPR30**
E2	0.65 (0.2–1.9)	9.0 (5–17)
E2-NH_3^+	4.1 (1.6–10)	25 (16–39)
E2-COO^-	2.0 (1.4–2.8)	30 (24–41)
E2-NMe_3^+	1.4 (0.5–3.9)	17 (14–22)
E2-NB	2.7 (1.2–5.8)	16 (10–25)

Although we could not detect any GPR30 at the cell surface, it was certainly possible that a small percentage, below our detection limit, is expressed and functions at the cell surface. To investigate whether small amounts of functional GPR30 may be expressed at the cell surface, we designed and synthesized a series of neutral and charged estrogen derivatives. We reasoned that if GPR30 were expressed in a functional state on the cell surface, both membrane-permeable and -impermeable estrogen derivatives should be capable of activating the receptor. However, if only an intracellular pool of GPR30 were present or functional, then only cell-permeable estrogen derivatives should be capable of activating the receptor.

The series of estrogen derivatives is shown (Figure 2). They are all based on the structure of 17α-ethynyl estradiol, a potent agonist of ERα/β and GPR30 that we have previously used to generate fluorescent estrogen derivatives. The three charged derivatives contain either an ionizable primary amino group (E2-NH_3^+) or carboxylate moiety (E2-COO^-) or alternatively a permanently charged quaternary amino group (E2-NMe_3^+). The fourth compound (E2-NB) is a neutral ^{t}Boc carbamate derivative of E2-NH_3^+. The E2-NH_3^+ and E2-COO^- derivatives are predicted to have pK_a values of 10.5 and 4.5, respectively, corresponding to $<$0.01% and 0.1% of the neutral species at physiological pH, which should be capable of crossing the cell membrane, resulting in slow intracellular accumulation. On the contrary, the permanently charged E2-NMe_3^+ is predicted not to cross the membrane to a significant extent. Finally, the neutral E2-NB derivative is expected to exhibit membrane permeability properties similar to those of E2 itself.

To assess the binding capacities of the novel estrogen derivatives toward ERα and GPR30, we utilized COS-7 cells, which lack both classical ERs and GPR30, and transfected them with either ERα–GFP or GPR30–GFP and performed competition-binding assays with Alexa633-labeled estrogen as a reporter (Figure 3). In this assay, permeabilization permits access of the charged fluores-

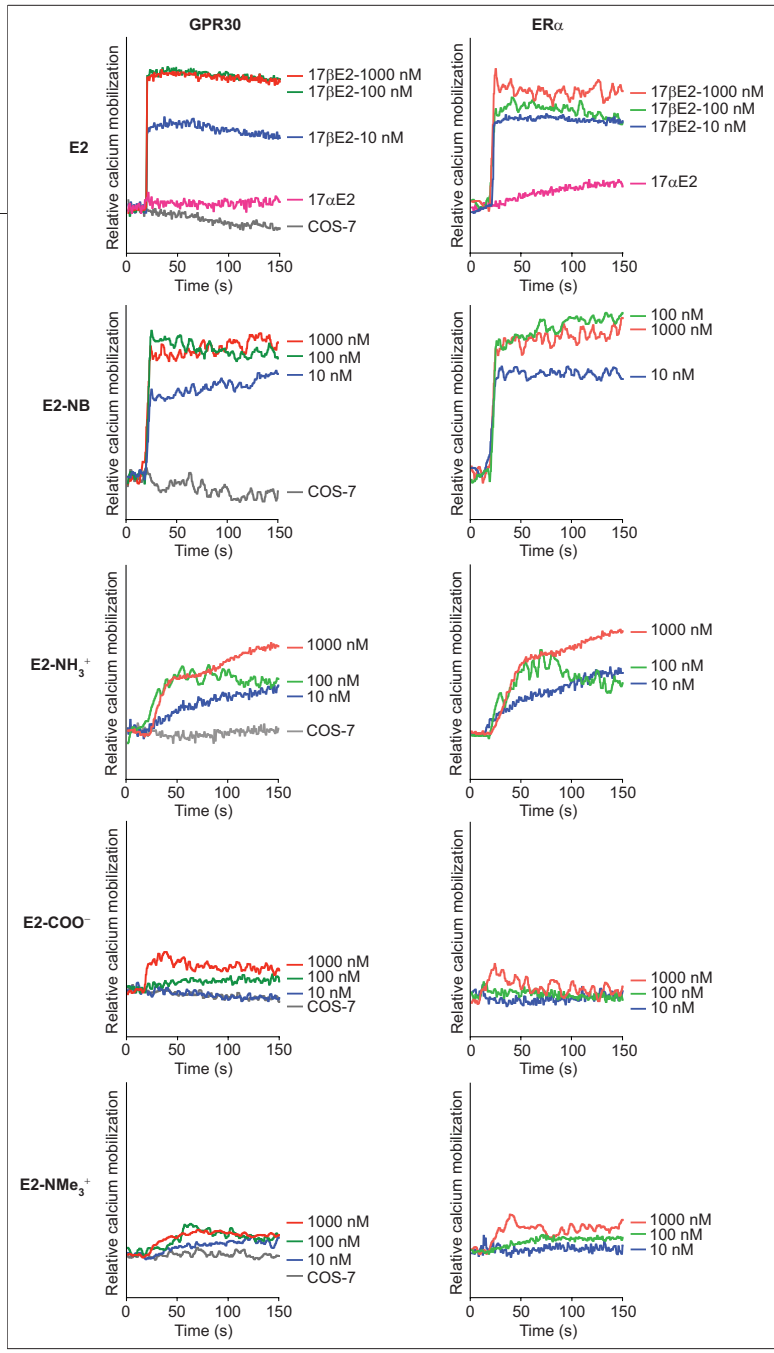

Figure 4. Calcium mobilization by E2 and the estrogen derivatives in GPR30- and ERα-expressing cells. COS-7 cells were transfected with either GPR30–GFP, ERα–GFP, or vector only, loaded with the calcium indicator, Indo1-AM, and assayed for alterations in intracellular calcium levels following stimulation with estrogen or the indicated estrogen derivative. 17α-Estradiol (17αE2) was used at 100 nM. Mock-transfected COS-7 cells (shown in the GPR30 panels, labeled COS-7) were treated with each ligand at 1000 nM, demonstrating that none of the estrogen derivatives exhibited receptor-independent effects. Data are representative of three independent experiments.

cent reporter and does not discriminate between permeable and impermeable ligands but simply measures the affinity of the compound for the given receptor. For ERα, where the K_i for E2 was 0.65 nM, the K_i values for the four estrogen derivatives varied from 1.4 to 4.1 nM (Table 1). For GPR30, where the K_i for E2 was 9.0 nM, the K_i values for the four estrogen derivatives varied from 16 to 30 nM (Table 1). Thus, for each receptor type, the E2 derivatives exhibited slightly lower affinities than E2 itself, but there was no significant difference among the E2 derivatives themselves. In all cases, the affinity of a given E2 derivative for GPR30 was reduced 10-fold as compared with ERα, consistent with the observed difference in affinity for E2 itself.

In order to examine the signaling capabilities of the neutral and charged estrogen derivatives, we initially chose to examine the very rapid cellular response of calcium mobilization, which occurs on the order of seconds. COS-7 cells were transfected with either ERα–GFP or GPR30–GFP as above. ERα-expressing cells were used as a control to assess the responses in cells known to express only intracellular ERs. As we have shown previously, when stimulated with E2, both ERα- and GPR30-transfected cells respond with an almost immediate and sustained rise in intracellular calcium (Figure 4). Comparison of the four estrogen derivatives revealed that the neutral E2-NB compound initiated calcium fluxes indistinguishable from those of E2. However, stimulation with the three charged derivatives produced either negligible (E2-COO^- and E2-NMe_3^+) or substantially slower (E2-NH_3^+) calcium rises for both ERα and GPR30.

The slow calcium response of ERα-expressing cells initiated by all concentrations (10–1000 nM) of E2-NH_3^+ suggests that this derivative is capable of cross-

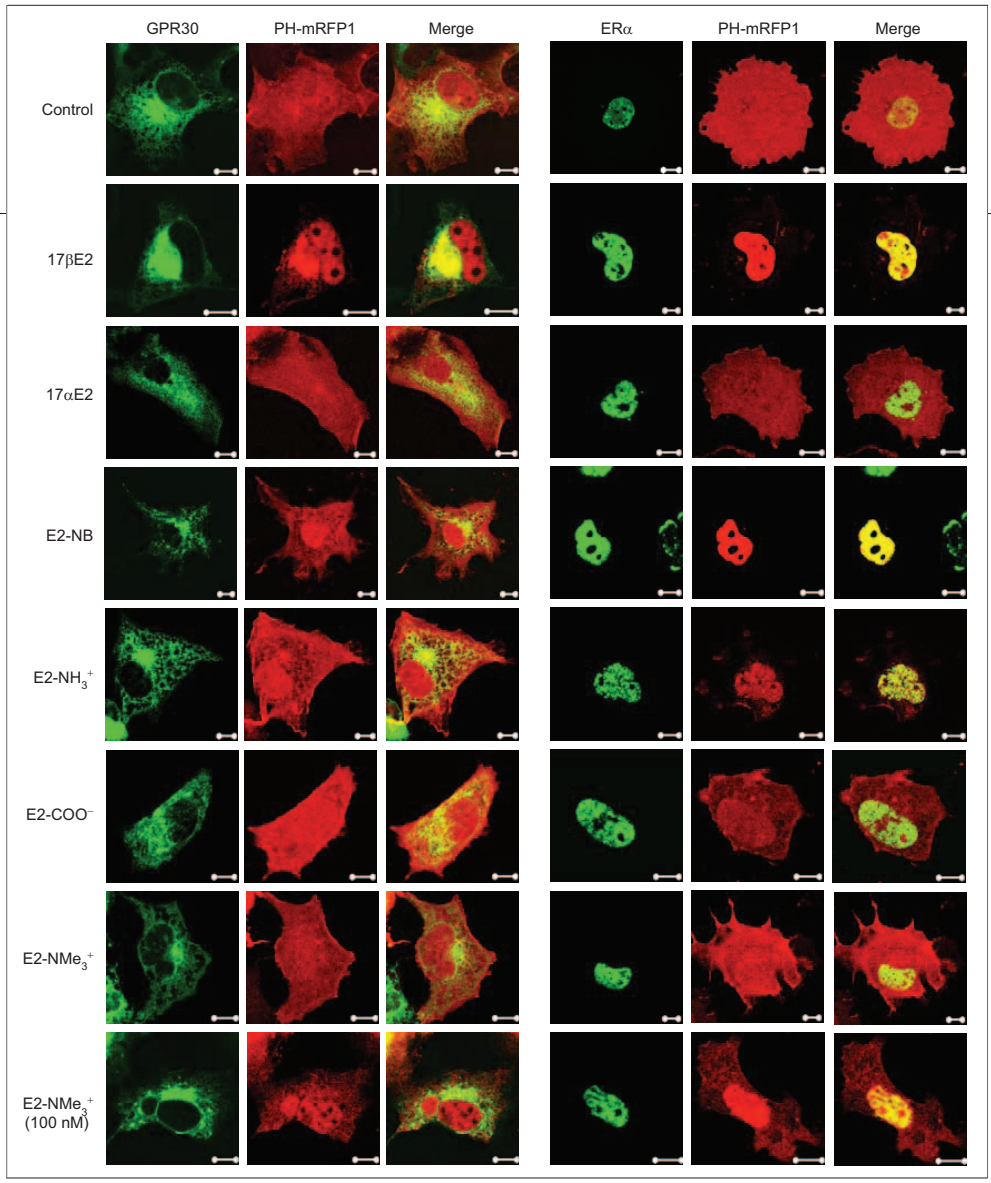

Figure 5. PIP3 production by GPR30- and ERα-expressing cells. COS-7 cells were transfected with PH–mRFP1 and either GPR30–GFP or ERα–GFP, starved overnight, and assayed for localization of the PH reporter following stimulation with estrogen (E2, 10 nM; 17αE2, 100 nM) or the indicated estrogen derivative (at 10 or 100 nM where indicated). The white bars denote 10 μm. Data are representative of three independent experiments.

ing the membranes slowly. On the contrary, little activation is observed with the negatively charged E2-COO^- derivative, perhaps due to unfavorable electrostatic interaction with the negatively charged components of the phospholipid bilayer. The juxtamembrane microenvironment may also result in the enhanced permeability of the positively charged E2-NH_3^+ derivative in its neutral form. Finally, the permanently charged E2-NMe_3^+ derivative exhibited almost no mobilization of calcium for either ERα or GPR30 even at concentrations 100-fold higher than that providing maximal activity for E2, suggesting that for both ERα and GPR30 intracellular access is essential for receptor activation.

To extend our analysis of the signaling capabilities of the novel estrogen derivatives, we examined the cellular activation of phosphoinositide 3-kinase (PI3K; Figure 5). We have previously shown that, in response to estrogen stimulation, both ERα and GPR30 mediate the nuclear accumulation of phosphatidylinositol 3,4,5-trisphosphate (PIP3) as assessed by the translocation of a chimeric Akt-PH-fluorescent protein reporter from the cytoplasm to the nucleus. In contrast to calcium mobilization, which occurs within seconds of stimulation, nuclear PIP3 accumulation requires 15 min of ligand stimulation for a clear effect to be observed. This longer time frame can give weakly permeable ligands used at high concentrations sufficient time to accumulate intracellularly and activate intracellular receptors. This was in fact observed in ERα-expressing cells as all ligands, when used at 100 nM, produced a response similar to estrogen at 10 nM (see bottom panel of Figure 5 for E2-NMe_3^+ at 100 nM). In ERα-expressing cells, at 10 nM, only E2-NB and E2-NH_3^+ produced a strong response. The E2-NMe_3^+ derivative produced virtually no response at 10 nM, whereas the E2-COO^- derivative produced a weak response. These observations are consistent with the calcium mobilization results where E2-NMe_3^+ and E2-COO^- produced little to no response but E2-NB and E2-NH_3^+ produced significant responses by the end of the 2 min calcium assay. In comparing the results of ERα-expressing cells with GPR30-expressing cells, we observed similar results, with GPR30 activation being mediated by 10 nM E2-NB and E2-NH_3^+ (as well as 100 nM E2-NMe_3^+) but not by 10 nM E2-NMe_3^+ and E2-COO^-.

The results described above demonstrate that GPR30 expressed in COS-7 cells can only be activated by ligands capable of crossing the plasma membrane. This suggests that intracellular GPR30 is functional and that there is insufficient GPR30 expressed on the plasma

membrane to initiate signaling by impermeable ligands. To test whether the same holds true for endogenously expressed GPR30, we determined the capabilities of the estrogen derivatives to activate PI3K in SKBr3 breast cancer cells, which endogenously express GPR30 but not the classical ERs (ERα/ERβ). As we have previously shown, in unstimulated SKBr3 cells, the Akt-PH reporter is localized to the plasma membrane due to constitutive activation of EGFR/erbB2 (Figure 6) (*16*). However, upon stimulation with E2, but not 17αE2, the reporter translocates to the nucleus. As with transfected COS-7 cells, stimulation of SKBr3 cells with E2-NB and E2-NH_3^+ activated GPR30 but stimulation with E2-NMe_3^+ and E2-COO^- did not, even at concentrations up to 100 nM. The lack of stimulation at 100 nM E2-NMe_3^+ and E2-COO^- in SKBr3 cells may be due to the lower level of GPR30 expression as compared with GPR30-transfected COS-7 cells.

Our interpretation of the above results is based on the predicted membrane permeability of the neutral, ionizable, and charged estrogen derivatives. To confirm the membrane permeability of these derivatives, we used an intact cell system with COS-7 cells expressing ERα as the sole intracellular estrogen-binding site. Specific binding for E2 was determined relative to untransfected COS-7 cells. Cells were incubated with estrogen or estrogen derivative for 5 min. Thereafter, tritiated estrogen was added for 5 min to bind to any unoccupied intracellular binding sites. Whereas E2 preincubation resulted in almost complete inhibition of tritiated estrogen binding (99% ± 3% competition relative to untransfected COS-7 cells), E2-NMe_3^+ displayed negligible competition (4.6% ± 4%), consistent with the predicted permeability of the two compounds (Figure 7). E2-NB (75% ± 3%), E2-NH_3^+ (69% ± 6%), and E2-COO^- (71% ± 3%) all displayed significant inhibition of tritiated estrogen binding, confirming that at high concentrations and over the extended length of this assay (~10 min), these compounds can gain access to the cell interior.

Recent evidence has begun to suggest the presence of functional intracellular GPCRs (*22, 28, 29*) and receptor tyrosine kinases (*30*). Studies have described the translocation of full-length EGFR and other receptor tyrosine kinases to the nuclear membrane of cancer cells following stimulation (*30*). In the case of GPCRs, evidence is accumulating that receptors for lipophilic agonists such as prostaglandins, platelet activating factor, and lysophosphatidic acid can be constitutively localized to the nuclear or perinuclear membrane where they may mediate intracrine signaling events (*22*). Based on these reports and our observations that GPR30 is expressed predominantly in the endoplasmic reticulum (*16*), we questioned whether this intracellular pool of receptor might be functionally active, particularly because its ligand E2 is freely permeable to cell membranes.

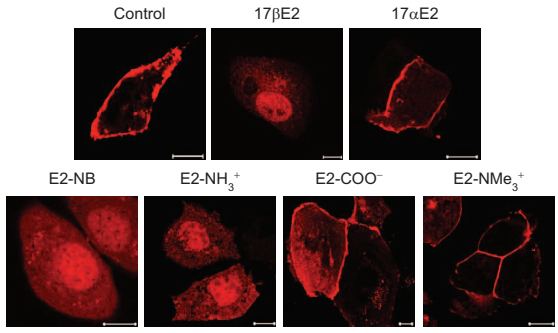

Figure 6. PIP3 production by endogenous GPR30 in SKBr3 cells. SKBr3 cells were transfected with PH–mRFP1, starved overnight, and assayed for localization of the PH reporter following stimulation with 100 nM estrogen or the indicated estrogen derivative for 15 min at 37 °C. Unstimulated cells show a pre-existing plasma membrane localization of the PH reporter due to the constitutive activity of EGFR and PI3K, resulting from erbB2 overexpression. Only upon stimulation with permeable ligands does the PH reporter translocate to the cell nucleus. The white bars denote 10 μm. Data are representative of three independent experiments.

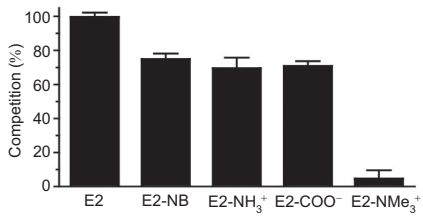

Figure 7. Access of estrogen derivatives to intracellular ERα. COS-7 cells were transfected with ERα–GFP, starved overnight, and assayed for tritiated estrogen binding. Cells were preincubated with 6 μM competitor (unlabeled estrogen, E2, E2-NH_3^+, E2-COO^-, E2-NMe_3^+, or E2-NB) for 5 min, after which an equal volume of tritiated estrogen was added for 5 min. Cell suspensions were then filtered through GF/C microfiber filters, washed twice, and counted in scintillation fluid. Data are means ± SEM of three independent experiments carried out in duplicate.

Because we have previously shown that the intracellular pool of GPR30 is capable of binding ligand, as demonstrated in permeabilized cells using fluorescent estrogen derivatives (*16*), we sought to use a collection of neutral and charged estrogen derivatives to probe the signaling capabilities of GPR30. Our results demonstrate that, although all the derivatives bind both ERα and GPR30 with affinities comparable to estrogen itself, only the compounds expected and demonstrated to be membrane-permeable are capable of rapidly activating GPR30 as assessed by calcium mobilization. This is most clearly seen with E2 itself and the E2-NB derivative, both of which lead to almost instantaneous increases in intracellular calcium concentrations. On the contrary, the permanently charged derivative E2-NMe_3^+ is incapable of rapidly activating either ERα or GPR30, even at concentrations >100× that required for E2 function. In contrast to the difference in rapid calcium signaling, both the E2-NB and E2-NMe_3^+ derivatives are equally capable of mediating estrogen responsive element-dependent reporter gene expression in MCF-7 cells over a 24 h period, though not as effectively as estrogen ($EC_{50} \approx 5$ nM for E2-NB and E2-NMe_3^+; $EC_{50} \approx 10$ pM for E2, unpublished data). This result is consistent with the activation of PI3K by high concentrations of charged derivatives. It further confirms that the structure of the linker distal to the estrogen moiety (*e.g.*, a charged quaternary amino group *vs* a neutral ^{t}Boc carbamate) has little effect on ER binding and activation, although the presence of linker itself substantially reduces transcriptional efficacy compared with estrogen.

The two ionizable derivatives E2-NH_3^+ and E2-COO^- yielded somewhat disparate though overall consistent results. The lack of a calcium response produced by E2-COO^- was consistent with the lack of activity seen with E2-NMe_3^+, suggesting that the rate of membrane permeabilization was reduced for both negatively and positively charged derivatives. However, stimulation with E2-NH_3^+ produced a slow increase in intracellular calcium, suggesting that this compound slowly crossed the membrane. The fact that the intracellular calcium concentration gradually increased for >2 min following administration, suggests that this compound enters the cell much more slowly than E2 or E2-NB. The exchangeable hydrogen-bonding capacity and favorable electrostatic interaction with anionic moieties of the phospholipid bilayer may explain the ability of this compound to cross the membrane. These small-molecule estrogen probes offer significant advantages for investigations of functional receptors compared with the impermeable macromolecular conjugate BSA-E2, where interpretation of membrane-associated ER-mediated responses is complicated by the presence of non-covalently associated E2 (*26*). Nevertheless, our studies have also shown a lack of BSA–E2-mediated calcium mobilization activity toward ER- and GPR30-expressing COS-7 cells consistent with our results reported here (unpublished observation).

Our results raise the question of how binding of a ligand to a GPCR in the endoplasmic reticulum could initiate signaling events, particularly those involving transactivation of EGFR. Because it has been shown that G protein βγ subunits are initially targeted to the endoplasmic reticulum, where they subsequently associate with G protein α subunits (*31*), it is likely that the necessary machinery for a GPCR to initiate signaling is present in the endoplasmic reticulum. Furthermore, although the signal transduction cascade initiated by GPR30 remains incompletely elucidated, GPCR-mediated transactivation of EGFR is commonly observed, occurring through multiple pathways, including Src-, protein kinase C-, or Ca^{2+}-mediated activation of cell-surface metalloproteinases, which then results in the release of EGF-like ligands (*8*). Because these latter intermediate signaling molecules are cytosolic, they are capable of diffusing from one membrane compartment to another.

Recent results from our lab examining GPR30 expression in endometrial tissues and cancers (*32*), as well as from Filardo *et al.* (*33*) examining GPR30 expression in breast tissue and cancer show that in human tissues, GPR30 staining yields uniform density throughout the cell, consistent with primarily intracellular localization. In contrast, our staining of the same tissues with anti-EGFR antibodies shows clear staining of the cell periphery, consistent with plasma membrane localization. Interestingly, Filardo *et al.* (*20*) have been able to isolate a subpopulation of amino-terminally hemagglutinin (HA)-tagged GPR30-transfected HEK293 cells that expresses GPR30 at the cell surface. This was accomplished by flow cytometric sorting of 1% of a transfected cell population stained with HA antibodies, suggesting the possibility that in a fraction of cells GPR30 can be expressed at the cell surface. However, examination of endogenously expressed GPR30 in SKBr3 cells by cellular fractionation showed that the vast majority of GPR30 protein is found in the microsomal (internal membrane) frac-

tion, consistent with our observations of intracellular staining. Intracellular expression of endogenous GPR30 in neurons has also recently been reported (*34*), although a contradictory report also exists (*19*). Thus, controversy still exists over the subcellular localization of GPR30.

In conclusion, our results provide the first evidence that a steroid-binding GPCR, GPR30, is capable of initiating signaling from an intracellular location. Further studies will be required to examine the regulation of subcellular distribution of GPR30, because other reports have observed GPR30 to be partially expressed on the cell surface, where it would also be capable of initiating signaling events. The possibility that signaling events initiated by GPR30 from these two distinct locations produce different cellular signals is an open question. Whether GPR30 localization in normal tissues and cells is regulated at the cellular level (possibly *via* cell stimulation or growth phase) or varies by tissue type or developmental/disease stage can now begin to be addressed with the novel estrogen-based reagents described in this study.

METHODS

Reagents. E2, 17αE2, Pluronic and Dulbecco's modified Eagle's medium (DMEM) were from Sigma. TO-PRO-3 and Alexa-conjugated secondary antibodies were from Molecular Probes. pEGFP-Actin was from Clontech. E2-2,4-[^{3}H] was from Sigma.

Synthesis of 17α-Phenylethynyl-E2 Derivatives (E2-NB, E2-NH_3^+, E2-NMe_3^+, and E2-COO^-). Full experimental procedures and compound characterization data are provided (see Supporting Information).

Receptor Expression. Cell lines were obtained from the ATCC. Cells were cultured in DMEM supplemented with 10% fetal calf serum. Transient transfections were performed 24 h after seeding cells using LipofectAMINE 2000 (Invitrogen) according to manufacturer's instructions. Cells were placed in serum-free and phenol-red-free DMEM/Ham's F-12 medium 16–48 h prior to experimentation.

Fluorescence Microscopy. Cells expressing GPR30 were seeded on 12 mm coverslips and fixed using 2% paraformaldehyde (PFA) in PBS, followed by blocking and permeabilization with 0.25% Triton X-100 in PBS with 3% BSA. Primary antibody, diluted in 3% goat serum, was incubated for 2 h at RT. Cells were subsequently washed three times with PBS and incubated with the appropriate secondary antibody diluted in 3% goat serum. The coverslips were washed three times with PBS and mounted using Vectashield. Confocal images were collected on a Zeiss LSM 510 confocal system.

Calcium Mobilization. Transfected 48–72 h serum-starved cells (5×10^6) were incubated at RT in Hank's balanced salt solution (HBSS) containing 5 μM Indo1-AM and 0.05% pluronic acid for 30 min. Cells were then washed once with HBSS and resuspended in HBSS at a density of 10^7 cells mL^{-1}. Ca^{2+} measurements were determined ratiometrically using $\lambda_{ex} = 340$ nm and $\lambda_{em} = 400/490$ nm at 37 °C in a spectrofluorometer (PTI QM-2000-2) equipped with a magnetic stirrer and water bath temperature control. The 490 nm/400 nm ratio was plotted as a function of time.

PI3K Activation. The PIP3 binding (PH) domain of Akt fused to mRFP1 (PH–mRFP1) was used to determine PIP3 localization and production by PI3K. COS-7 cells were cotransfected with PH–mRFP1 and either GPR30–GFP or ERα–GFP. Cells were plated on coverslips and serum-starved for 24 h prior to use. The cells on coverslips were fixed with 2% PFA in PBS, washed, mounted with Vectashield, and visualized by confocal microscopy.

Tritiated Estrogen Binding to ER. Confluent COS-7 cells were transfected with ERα–GFP using Lipofectamine 2000 and serum-starved overnight. Cells were trypsinized and washed 3 times in serum-free F12/DMEM to remove trypsin. Cells ($(4-8) \times 10^5$) were incubated in 50 μL of F12 containing 6 μM competing compounds for 5 min at RT, after which 50 μL of 12 nM [^{3}H]-E2 in F12 was added. Tritiated estrogen binding was allowed to occur for an additional 5 min at RT, after which the cell suspension was applied to GF/C microfiber filters, washed twice with 2 mL of PBS each, and counted in scintillation fluid.

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Supporting Information Available: This material is free of charge *via* the Internet.

REFERENCES

1. Thomas, T., Gallo, M. A., and Thomas, T. J. (2004) Estrogen receptors as targets for drug development for breast cancer, osteoporosis and cardiovascular diseases, *Curr. Cancer Drug Targets 4*, 483–499.
2. O'Dowd, B. F., Nguyen, T., Marchese, A., Cheng, R., Lynch, K. R., Heng, H. H., Kolakowski, L. F., Jr., and George, S. R. (1998) Discovery of three novel G-protein-coupled receptor genes, *Genomics 47*, 310–313.
3. Owman, C., Blay, P., Nilsson, C., and Lolait, S. J. (1996) Cloning of human cDNA encoding a novel heptahelix receptor expressed in Burkitt's lymphoma and widely distributed in brain and peripheral tissues, *Biochem. Biophys. Res. Commun. 228*, 285–292.
4. Takada, Y., Kato, C., Kondo, S., Korenaga, R., and Ando, J. (1997) Cloning of cDNAs encoding G protein-coupled receptor expressed in human endothelial cells exposed to fluid shear stress, *Biochem. Biophys. Res. Commun. 240*, 737–741.
5. Carmeci, C., Thompson, D. A., Ring, H. Z., Francke, U., and Weigel, R. J. (1997) Identification of a gene (GPR30) with homology to the G-protein-coupled receptor superfamily associated with estrogen receptor expression in breast cancer, *Genomics 45*, 607–617.

6. Filardo, E. J., Quinn, J. A., Bland, K. I., and Frackelton, A. R., Jr (2000) Estrogen-induced activation of Erk-1 and Erk-2 requires the G protein-coupled receptor homolog, GPR30, and occurs *via* transactivation of the epidermal growth factor receptor through release of HB-EGF, *Mol. Endocrinol. 14*, 1649–1660.
7. Filardo, E. J., Quinn, J. A., Frackelton, A. R., Jr., and Bland, K. I. (2002) Estrogen action *via* the G protein-coupled receptor, GPR30: stimulation of adenylyl cyclase and cAMP-mediated attenuation of the epidermal growth factor receptor-to-MAPK signaling axis, *Mol. Endocrinol. 16*, 70–84.
8. Fischer, O. M., Hart, S., Gschwind, A., and Ullrich, A. (2003) EGFR signal transactivation in cancer cells, *Biochem. Soc. Trans. 31*, 1203–1208.
9. Maggiolini, M., Vivacqua, A., Fasanella, G., Recchia, A. G., Sisci, D., Pezzi, V., Montanaro, D., Musti, A. M., Picard, D., and Ando, S. (2004) The G protein-coupled receptor GPR30 mediates c-fos up-regulation by 17beta-estradiol and phytoestrogens in breast cancer cells, *J. Biol. Chem. 279*, 27008–27016.
10. Kanda, N., and Watanabe, S. (2003) 17β-Estradiol enhances the production of nerve growth factor in THP-1-derived macrophages or peripheral blood monocyte-derived macrophages, *J. Invest. Dermatol. 121*, 771–780.
11. Kanda, N., and Watanabe, S. (2003) 17β-Estradiol inhibits oxidative stress-induced apoptosis in keratinocytes by promoting Bcl-2 expression, *J. Invest. Dermatol. 121*, 1500–1509.
12. Ylikomi, T., Vienonen, A., and Ahola, T. M. (2004) G protein-coupled receptor 30 down-regulates cofactor expression and interferes with the transcriptional activity of glucocorticoid, *Eur. J. Biochem. 271*, 4159–4168.
13. Ahola, T. M., Manninen, T., Alkio, N., and Ylikomi, T. (2002) G protein-coupled receptor 30 is critical for a progestin-induced growth inhibition in MCF-7 breast cancer cells, *Endocrinology 143*, 3376–3384.
14. Vivacqua, A., Bonofiglio, D., Albanito, L., Madeo, A., Rago, V., Carpino, A., Musti, A. M., Picard, D., Ando, S., and Maggiolini, M. (2006) 17β-Estradiol, genistein and 4-hydroxytamoxifen induce the proliferation of thyroid cancer cells through the G protein-coupled receptor GPR30, *Mol. Pharmacol. 70*, 1414–1423.
15. Vivacqua, A., Bonofiglio, D., Recchia, A. G., Musti, A. M., Picard, D., Ando, S., and Maggiolini, M. (2006) The G protein-coupled receptor GPR30 mediates the proliferative effects induced by 17β-estradiol and hydroxytamoxifen in endometrial cancer cells, *Mol. Endocrinol. 20*, 631–646.
16. Revankar, C. M., Cimino, D. F., Sklar, L. A., Arterburn, J. B., and Prossnitz, E. R. (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling, *Science 307*, 1625–1630.
17. Thomas, P., Pang, Y., Filardo, E. J., and Dong, J. (2005) Identity of an estrogen membrane receptor coupled to a G-protein in human breast cancer cells, *Endocrinology 146*, 624–632.
18. Filardo, E. J. (2002) Epidermal growth factor receptor (EGFR) transactivation by estrogen *via* the G-protein-coupled receptor, GPR30: a novel signaling pathway with potential significance for breast cancer, *J. Steroid Biochem. Mol. Biol. 80*, 231–238.
19. Funakoshi, T., Yanai, A., Shinoda, K., Kawano, M. M., and Mizukami, Y. (2006) G protein-coupled receptor 30 is an estrogen receptor in the plasma membrane, *Biochem. Biophys. Res. Commun. 346*, 904–910.
20. Filardo, E., Quinn, J., Pang, Y., Graeber, C., Shaw, S., Dong, J., and Thomas, P. (2007) Activation of the novel estrogen receptor, GPR30, at the plasma membrane. *Endocrinology*, in press.
21. Innamorati, G., Piccirillo, R., Bagnato, P., Palmisano, I., and Schiaffino, M. V. (2006) The melanosomal/lysosomal protein OA1 has properties of a G protein-coupled receptor, *Pigment Cell Res. 19*, 125–135.
22. Gobeil, F., Fortier, A., Zhu, T., Bossolasco, M., Leduc, M., Grandbois, M., Heveker, N., Bkaily, G., Chemtob, S., and Barbaz, D. (2006) G-protein-coupled receptors signalling at the cell nucleus: an emerging paradigm, *Can. J. Physiol. Pharmacol. 84*, 287–297.
23. Lu, M., Echeverri, F., and Moyer, B. D. (2003) Endoplasmic reticulum retention, degradation, and aggregation of olfactory G-protein coupled receptors, *Traffic 4*, 416–433.
24. Muller, R. E., Johnston, T. C., Traish, A. M., and Wotiz, H. H. (1979) Studies on the mechanism of estradiol uptake by rat uterine cells and on estradiol binding to uterine plasma membranes, *Adv. Exp. Med. Biol. 117*, 401–421.
25. Bologa, C. G., Revankar, C. M., Young, S. M., Edwards, B. S., Arterburn, J. B., Kiselyov, A. S., Parker, M. A., Tkachenko, S. E., Savchuck, N. P., Sklar, L. A., Oprea, T. I., and Prossnitz, E. R. (2006) Virtual and biomolecular screening converge on a selective agonist for GPR30, *Nat. Chem. Biol. 2*, 207–212.
26. Stevis, P. E., Deecher, D. C., Suhadolnik, L., Mallis, L. M., and Frail, D. E. (1999) Differential effects of estradiol and estradiol-BSA conjugates, *Endocrinology 140*, 5455–5458.
27. Taguchi, Y., Koslowski, M., and Bodenner, D. L. (2004) Binding of estrogen receptor with estrogen conjugated to bovine serum albumin (BSA), *Nucl. Recept. 2*, 5.
28. Marrache, A. M., Gobeil, F., Zhu, T., and Chemtob, S. (2005) Intracellular signaling of lipid mediators *via* cognate nuclear G protein-coupled receptors, *Endothelium 12*, 63–72.
29. Zhu, T., Gobeil, F., Vazquez-Tello, A., Leduc, M., Rihakova, L., Bossolasco, M., Bkaily, G., Peri, K., Varma, D. R., Orvoine, R., and Chemtob, S. (2006) Intracrine signaling through lipid mediators and their cognate nuclear G-protein-coupled receptors: a paradigm based on PGE2, PAF, and LPA1 receptors, *Can. J. Physiol. Pharmacol. 84*, 377–391.
30. Lo, H. W., and Hung, M. C. (2006) Nuclear EGFR signalling network in cancers: linking EGFR pathway to cell cycle progression, nitric oxide pathway and patient survival, *Br. J. Cancer 94*, 184–188.
31. Michaelson, D., Ahearn, I., Bergo, M., Young, S., and Philips, M. (2002) Membrane trafficking of heterotrimeric G proteins *via* the endoplasmic reticulum and Golgi, *Mol. Biol. Cell 13*, 3294–3302.
32. Smith, H. O., Leslie, K. K., Singh, M., Qualls, C. R., Revankar, C. M., Joste, N. E., and Prossnitz, E. R. (2007) GPR30: A novel indicator of poor survival for endometrial carcinoma, *Am. J. Obstet. Gynecol. 196*, 386.e381–386.e311.
33. Filardo, E. J., Graeber, C. T., Quinn, J. A., Resnick, M. B., Giri, D., DeLellis, R. A., Steinhoff, M. M., and Sabo, E. (2006) Distribution of GPR30, a seven membrane-spanning estrogen receptor, in primary breast cancer and its association with clinicopathologic determinants of tumor progression, *Clin. Cancer Res. 12*, 6359–6366.
34. Brailoiu, E., Dun, S. L., Brailoiu, G. C., Mizuo, K., Sklar, L. A., Oprea, T. I., Prossnitz, E. R., and Dun, N. J. (2007) Distribution and characterization of estrogen receptor G protein-coupled receptor 30 in the rat central nervous system, *J. Endocrinol. 193*, 311–321.