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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

We have investigated the interactions between estrogen and caveolin proteins in breast cancer. There is a physical interaction between caveolin-1 and the plasma membrane estrogen receptor in cultured breast cancer cells that is strongly downregulated by estradiol in 30 minutes. This leads to enhanced ERK activation and proliferation of the cells. Overexpressing caveolin-1 leads to a downregulation of the ability of estradiol to activate the ERK (MAP kinase) signal pathway. Estrogen also inhibits caveolin-1 synthesis in breast cancer cells. Caveolin-1 facilitates ER localization to the plasma membrane, demonstrated in breast cancer cells (MCF-7). As for the structure/function of the plasma membrane estrogen receptor, expression and targeting of only the E domain (ligand binding) to the plasma membrane is sufficient for estrogen signaling to ERK. This work was published in Mol Endocrinology 16(1):100-115, 2002.

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Table of Contents

Cover.....1

SF 298.....2

Introduction.....4

Body.....4

Key Research Accomplishments.....4

Reportable Outcomes.....5

Conclusions.....5

References.....5

Appendices.....6

Introduction

Estrogen promotes an increased incidence of breast cancer in women, while anti-estrogen therapy both limits recurrences and prevents the development of primary disease in genetically predisposed individuals. The purpose of the studies proposed in our grant is to determine the cellular mechanisms by which estrogen enhances the survival of breast cancer.

Body

In conjunction with the approved "statement of work", we continue to explore the mechanisms by which acts as a survival and growth factor for breast cancer. We examined the interactions between the membrane domain protein, caveolin-1, and the plasma membrane estrogen receptor (ER). We found that in the basal state, ER associates with caveolin-1 in the membrane and in caveolae fractions of endothelial cells, breast cancer cells and vascular smooth muscle cells. In breast cancer, estradiol (E_2) strongly and rapidly (30 minutes) downregulates ER/Caveolin-1 physical association, in an ERK (Map kinase)-dependent fashion. E_2 also significantly decreases the synthesis and stability of caveolin-1 in breast cancer cells. This leads to enhanced E_2-induced ERK activation, important for breast cancer cell survival and proliferation. Transient transfection and expression of caveolin-1 in breast cancer leads to a decreased ability of E_2 to activate ERK. Caveolin-1 also facilitates ER translocation to the plasma membrane. Regarding structure/function, targeting the E domain (hormone binding) domain of ERα to the plasma membrane resulted in ERK activation, while targeting the E domain to the nucleus had no effect on ERK. This suggests that the E domain is the important part of the membrane ER that allows for the interactions that trigger ERK activation. This work was published in Molecular Endocrinology 16(1):100-115, 2002.

We then investigated how Estrogen signals from the plasma membrane receptor to promote the survival and proliferation of breast cancer cells. We found that E_2 stimulates Giα, Gqα, and Gβδ proteins to stimulate calcium and PKC, via phospholipase C activation. This led to Src-dependent activation of matrix metalloproteinases 2 and 9, secretion of heparin-binding EGF, transactivation of the EGF receptor, and signaling to ERK and AKT kinases in breast cancer. This work was recently submitted to JBC.

Key Research Accomplishments

- Identification of how estrogen receptors move to the plasma membrane and signal in breast cancer.
- Understanding the different effects of estrogen in different cell types.
- Elucidation of the important cross-talk between plasma membrane ER and the EGF receptor, resulting in breast cancer proliferation.

Reportable Outcomes

Abstracts and Presentations

1. Razandi M, Oh P, Pedram A, Schnitzer J, Levin ER. Estrogen associates with and modulates caveolin production. Impact for cell signaling. Presented at the 83rd Annual Meeting of the Endocrine Society, Denver, CO, June 2001.

2. Razandi M, Alton G, Pedram A, Shonshani S, Levin ER. Serine 522 of mouse estrogen receptor alpha is essential for membrane localization, signaling and cell biology. Presented at the 84th Annual Meeting of the Endocrine Society, San Francisco, CA, June 2002.

3. Pedram A, Razandi M, Park ST, Levin ER. Proximal events in membrane estrogen receptor signaling requires G-protein induced transactivation of the EGF receptor. Presented at the 84th Annual Meeting of the Endocrine Society, San Francisco, CA, June 2002.

Manuscripts

1. Kelly M, Levin ER. Rapid actions of plasma membrane estrogen receptors. Trends Endocrinol Metab 12(4):152-156, 2001.

2. Levin ER. Cell localization, physiology and nongenomic actions of estrogen receptors. J Applied Physiol 91:1860-1867, 2001.

3. Levin ER. Cellular functions of plasma membrane estrogen receptors. Steroids 67(6):471-475, 2002.

4. Razandi M, Oh P, Pedram A, Schnitzer J, Levin ER. Estrogen receptors associate with and regulate the production of caveolin: Implications for signaling and cellular actions. Mol Endocrinol 16:100-115, 2002.

Conclusions

Estradiol signaling from the membrane ER is important for cell survival and proliferation in breast cancer. This occurs through functional interactions with caveolin-1 and the EGF receptor. Furthermore, the membrane ER is localized to the cell surface through interactions with caveolin-1. This justifies estrogen antagonist synthesis that only works at the cell surface, to help prevent breast cancer.

References

None.

Appendices
Three articles attached.

J Appl Physiol
91: 1860–1867, 2001.

highlighted topics

Genome and Hormones: Gender Differences in Physiology Invited Review: Cell localization, physiology, and nongenomic actions of estrogen receptors

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Levin, Ellis R. Invited Review: Cell localization, physiology, and nongenomic actions of estrogen receptors. *J Appl Physiol* 91: 1860–1867, 2001.—The existence of binding proteins for the female sex steroid, 17β-estradiol, has been known for almost 50 years. Presently, two estrogen receptors (ERs), ER-α and ER-β, have been cloned in mammals, and they are expressed in many cell types of metazoans. ERs act primarily as nuclear transcription factors, and this effect is enhanced by ligand binding. Emerging data have identified a separate pool of receptors for this steroid in the plasma membrane, but the mechanisms of action and cellular functions of these proteins are just beginning to be defined. In this review, the known details of the nuclear and plasma membrane ER functions will be discussed. A particular focus will be to define the signaling pathways from the membrane that lead to important cell physiology effects of estrogen. The potential interactions of membrane ER with other local proteins will also be discussed, and the unique but often complementary roles of the receptor pools will be highlighted. These details may be of additional relevance to other steroid receptors, since there is evidence of their existence in the cell membrane.

nuclear receptors; apoptosis; membrane receptors; steroid receptors; signal transduction; cell proliferation

ESTROGEN RECEPTORS (ERs) mediate the important actions of the endogenous steroid hormone, 17β-estradiol (E_2), and thereby participate in various aspects of cellular physiology. ER is synthesized in many cell types as two protein forms, ER-α (19, 20) and ER-β (30, 48, 79), which are the products of separate genes. Alternatively spliced transcripts for the receptors largely account for the differential length binding proteins in discrete cell types. In many cells, the receptors coexist either as homodimers or as heterodimers (11, 55), but the distribution of the two receptors does not completely overlap. ER-α exists as the predominant receptor in most target organs (38). However, ER-β is prominently expressed in ovary, prostate, lung, and hypothalamus (9, 31), and recent evidence indicates that there are specific actions of E_2 that can be attributed to one receptor but not the other (30, 70, 81). The actions of E_2 occur on binding ER, and at least the nuclear pool of these receptors then transactivates relevant genes (17, 21). In some circumstances, the ER subtypes differentially transactivate target genes that participate in the cell biological effects of the steroid hormone (52, 76, and reviewed in Refs. 50 and 67).

Gene deletion of each of the two receptor proteins has revealed the importance of E_2 for normal female sex organ development and function (10, 30, 38). Both in vivo and in vitro studies in mammals have suggested that E_2 has significant actions for the preservation of bone (74) and blood vessel integrity (44, 71) and contributes to brain function (42) and the modulation of immunity (86). Estrogen also appears to be a significant growth and survival factor for human breast cancer cells (45, 64). Several of the actions of estrogen (as well as other steroid hormones) occur rapidly and

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are therefore considered to be nongenomic (reviewed in Ref. 35). These effects include the enactment of signal transduction originating from the plasma membrane.

Insight into the rapid, nongenomic actions of E_2 began to take substance more than 20 years ago when a second pool of ERs was identified (57, 58). Subsequent work has begun to clarify the location and function of this binding protein in the cell plasma membrane (7, 8, 63, 68). The membrane receptor(s) has not been isolated or sequenced to date, but there appears to be ER-α that is structurally very similar to the nuclear receptor in many cell types (53). The membrane ERs probably also include ER-β (63). Various signal transduction pathways have been recently identified to be rapidly triggered by E_2, and these contribute to the cell biological effects of this steroid.

NUCLEAR RECEPTORS

The identification of receptors for E_2 was most advanced by work from the laboratories of Jensen and Gorski (4, 23, 78). Subsequent purification of the binding protein (now known as ER-α) resulted in antibodies being generated (19), and this led to the localization of receptors in the nucleus of target cells for E_2 action (28). The cDNA for the human ER-α was cloned in 1985 (82); in the 1990s, evidence of complex interactions with coactivator proteins and basal transcriptional machinery proteins was provided (reviewed in Ref. 40) (Fig. 1).

An important discovery of a second ER, ER-β, was reported in 1996 (32, 48, 79). ER-α and ER-β are individually well conserved through mammalian species (54, 79), and there is evidence of a newly identified receptor, ER-γ, in teleost fish (22). The two nuclear receptors are homologous mainly in their DNA binding and ligand pocket binding domains but less so in their respective hinge and ligand-independent activation function (AF-1) region. Alternative splicing of the mRNA of ER-β gives rise to several protein isoforms, and a recently described 46-kDa ER-α isoform may be important in breast cancer (17).

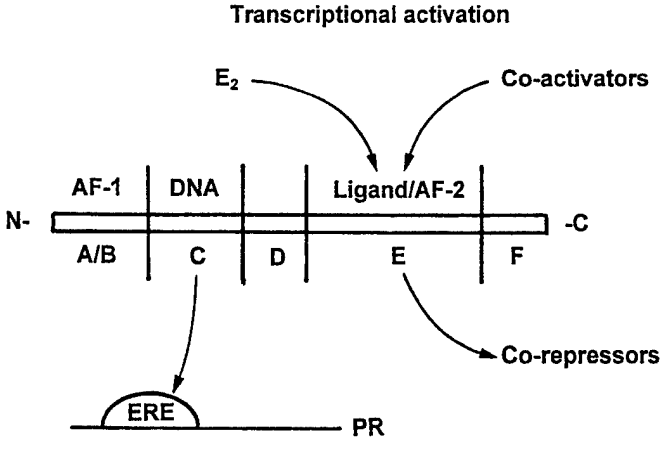

Fig. 1. The nuclear estrogen receptor (ER) contains A through F domains, with activation function (AF)-1 (ligand independent) and AF-2 (ligand dependent) facilitating the transactivation of target genes. ERE, estrogen response element; E_2, 17β-estradiol; PR, progesterone receptor; N, NH_2 terminus; C, COOH terminus.

The steroid receptor translocates to the nuclear membrane from the cytosol in a ligand-independent fashion (54) and can activate transcription independently of ligand. However, ligand serves to recruit coactivator proteins (and leads to the displacement of corepressors), functioning mainly through the AF-2 domain of the receptors. The transcriptional effects of the nuclear receptors can be mediated through several mechanisms. E_2-ER complexes bind classical inverse palindromic (CAGGTCAnnnTGACCTGA) or nonclassical estrogen response elements on the promoters of target genes. Alternatively, E_2-ER complexes transactivate genes through protein-protein interactions with *1*) transcription factors such as activator protein 1 or Sp-1 that bind DNA, *2*) coaccessory proteins (Src, ACTR), some of which have histone acetylase activity, and *3*) RNA polymerase II complex proteins (43). There is evidence that ligation of the two nuclear receptors can differentially transactivate genes (52) and, as mentioned, that the receptors can form heterodimers in vivo (11, 55). In addition, ERs serve to repress genes (80), and this also plays important roles in E_2 action.

Traditionally, many of the functions of E_2 are proposed to be mediated through target gene upregulation, considered to be the main function of the nuclear ER (reviewed in Ref. 10). This is most apparent in sex organ development and function in females. ER-α also has an important but unanticipated role in fertility and sperm maturation and function, as demonstrated in the ER-α knockout male mouse (14). Estrogen also affects central nervous system functions and the neuroendocrine-gonadal axis in both sexes and seems to preserve bone density (10). Thus the loss of estrogen action after menopause is considered a major risk factor in the development of osteoporosis in women (reviewed in Ref. 62). There is also epidemiological and experimental data supporting the importance of estrogen to prevent atherosclerosis (44, 75) and to affect immunity (41, 86). Estrogen promotes breast cancer propagation (69) and mediates complex sexual behaviors, at least in rodents (56). In some of these situations, the genes that serve as targets for transactivation by the nuclear ER and participate in estrogen-induced cell biology have been identified. However, in many instances, the important target genes are unknown. Another caveat is the recent identification of membrane ER (see below). Because E_2 can induce rapid signaling from the membrane, it is likely that the cell biology of estrogen action is more complex than originally anticipated by invoking the nuclear model. Determining the relative contributions by each of these two receptor pools is an important focus in understanding the overall actions of the sex steroid.

MEMBRANE RECEPTORS

Background. Pietras and Szego (57, 58) originally described an E_2-binding protein in cell membranes

that triggered the rapid generation of cAMP. Subsequent work from many investigators indicated that E_2 rapidly activates signaling, such as calcium flux (77), phospholipase C activation (34), and inositol trisphosphate (IP_3) generation (36). In most studies, these actions appear to require E_2 binding to ERs. In neural cells, ERs can activate protein kinase C and protein kinase A and uncouple opioidergic and gabanergic receptors from their effector signaling molecules (reviewed in Ref. 27). These signaling events are likely to arise from the activation of G proteins by E_2, and this was directly shown for $G_s\alpha$ and $G_q\alpha$ in Chinese hamster ovary (CHO) cells expressing either subtype of ER (63). Thus ERs appear to be part of the large family of G-protein-coupled receptors (GPCR). After several G proteins are activated, E_2-ER can then trigger signaling cascades that culminate in a cell biological function.

Signaling pathways activated by estrogen and implications for cell physiology. An important pathway for E_2 action is the stimulation of the proline-directed, threonine/serine kinase, extracellular-regulated kinase (ERK). This member of the mitogen-activated protein (MAP) kinase family is rapidly (5 min) activated by E_2 and results from more proximal kinase activation, including Ras, Src, raf, and MAP kinase kinase stimulation in MCF-7 breast cancer cells (45). ERK activation via this cascade contributes to E_2-induced proliferation (6) and survival of MCF-7 cells (64) and prostate cancer cell proliferation (46), whereas specific ER antagonists such as ICI-182780 inhibit E_2 activation of this (and many) signals. Recently, Kousteni et al. (29) showed that E_2 signaled through the same pathway, to the survival of osteoblasts. Interestingly, osteoblast survival could also be shown in HeLa cells, mediated by targeting the E domain of ER-α to the cell membrane (but not to the nucleus), suggesting the role of the plasma membrane ER in this cell action. ERK activation by E_2 also underlies the stimulation of nitric oxide production in endothelial cells (ECs) and prevents glutaminergic, excitotoxicity-induced neuronal necrosis (72). Augmentation of ERK may also induce the activation of immediate early genes such as c-*fos* (84), which then transactivate other genes that are important for the cell biological effects of this steroid.

Signaling may also more directly activate E_2-responsive target genes. For instance, E_2-induced prolactin gene transcription requires signaling through ERK (85). By activating kinases, the membrane ER can participate in a transactivation function that was felt to be the exclusive domain of the nuclear ER (18, 21). A similar scenario has been established for a variety of growth factors that activate ERK and thus phosphorylate the nuclear ER for instance at serine 118 (5). ERK-induced phosphorylation stimulates the transactivation function of ER in an E_2-independent fashion (25). It has also been shown that nuclear receptor coactivator proteins can be phosphorylated by ERK and that this enhances steroid receptor transcriptional functions (66). These different substrates for phosphorylation by ERK may in part contribute to the important cross talk between growth factor receptor tyrosine kinases, such as the epidermal growth factor receptor, and ERs in target cells (12), including breast cancer cells. Regulation by signaling from the membrane also extends to the repression of genes. E_2 suppresses the activator protein 1-mediated transactivation of the preproendothelin-1 gene, induced by angiotensin II via ERK signaling in ECs (47).

Additional signal transduction pathways originating from the membrane have recently been identified as being rapidly responsive to E_2. In ECs, this sex steroid (as well as glucocorticoid and thyroid hormone) stimulates phosphoinositol-3-hydroxy kinase (71). This leads to the activation of Akt kinase and the generation of nitric oxide and is inhibited by ICI-182780. In an in vivo model of muscle injury following ischemia-reperfusion, this pathway was responsible for the ability of E_2 to prevent leukocyte accumulation (71). Also in ECs, E_2 activates the p38β isoform of the MAP kinase family, leading to activation of the threonine/serine MAPKAP-2 kinase and the phosphorylation of heat shock protein 27 (HSP27) (65). By expressing dominant-negative mutants of these three molecules, E_2 was shown to utilize this pathway to protect ECs from metabolic disruption of the actin cytoskeleton and hypoxia-induced cell death and to stimulate angiogenesis.

In ER-expressing CHO cells, it was also demonstrated that E_2 activates a third MAP kinase, c-Jun NH_2-terminal kinase (JNK), via ER-β but inhibits this kinase via ER-α (63). These results indicate that the two subtypes of ER can differentially modulate signaling pathways. The physiological relevance of this observation was recently demonstrated in breast cancer cells. Both chemotherapy and radiation treatment kill cells mainly by inducing apoptosis via a JNK-dependent mechanism. It has recently been shown that E_2 rapidly blocks JNK activation in this setting, preventing the JNK-induced, inactivating phosphorylation of Bcl-2 and Bcl-xl proteins, the subsequent stimulation of the caspase cascade, and cell death (64). In this way, E_2 can act as a survival factor, initiated through membrane signaling. Interestingly, tamoxifen, an estrogen antagonist that prevents the primary occurrence or recurrence of breast cancer in women, activates apoptosis of breast cancer cells through a JNK-dependent mechanism (39). With regard to the regulation of JNK activity, the ability of E_2 to prevent osteoclast formation in bone stems from the inhibition of regulating receptor activation of NF-κB ligand-induced JNK activation. This is likely to occur from a membrane ER expressed on monocytes (74).

Localization of membrane ER. To better understand the function of the membrane ER, we need to know the physical structure of the receptor and where it resides within the lipid bilayer. The endogenous receptor has not yet been isolated and sequenced. However, we know that a variety of antibodies directed against multiple epitopes of the nuclear ER-α identify an endogenous membrane protein in several cell types (53). In addition, expression in CHO cells of a single cDNA

for ER-α results in both membrane and nuclear pools of receptors (63). Therefore, it appears that the membrane receptor must be very similar to the nuclear receptor. Recent work has begun to clarify the location of this receptor within the plasma membrane. Signaling by growth factor receptors and non-growth factor tyrosine kinases as well as G protein receptors occurs at least in part after localization to plasma membrane microstructures, known as caveolae (1). This organelle facilitates signal transduction through the localization of signaling molecules (51), and this interaction is dependent on the high-cholesterol content and a structural coat protein family, the caveolins. It is believed that caveolin-1 can serve as a scaffold protein, associating with a variety of signaling molecules to organize their activation within the caveolae domains. Although caveolin indirectly facilitates signaling, it may directly inhibit various signal molecules. It is appreciated that caveolin-1 physically associates with endothelial nitric oxide synthase (eNOS). After calcium activation, calmodulin competitively displaces caveolin-1 from binding to eNOS (15) and caveolin-1 moves out of the membrane (26). These events are necessary for the activation of eNOS. Recently, ER has been shown to localize mainly to caveolae but also to noncaveolar fractions of the EC plasma membrane (7, 26). It is primarily within caveolae that E_2 activates eNOS after E_2-ER binding (7). We recently found that caveolin-1 physically associates with ERs in several cell compartments and that caveolin may impact the ability of ERs to both signal and localize to the plasma membrane (Razandi M, Pedram A, and Levin ER, unpublished observations).

Complementary role of membrane and nuclear ER. Although ERs in the membrane and nuclear compartments appear to act by very different mechanisms (signaling vs. transcriptional transactivation), the cell biological roles may overlap or be complementary. As mentioned, there is precedent for E_2 to activate gene transcription from both receptor pools. One may envision that kinase signaling can rapidly activate transcription, which can then be sustained by the nuclear receptor. As mentioned, the latter's action is probably facilitated by the phosphorylation of coactivator proteins, and this could result from ER signaling from the membrane. Signaling from the membrane may also amplify the actions of the nuclear receptor. Furthermore, signaling appears to play an important role in the posttranslational modification of proteins that can be upregulated in their synthesis via the nuclear receptor.

One example of this is the important anti-apoptotic protein, Bcl-2. It is well described that this gene can be activated by E_2, in part through an Sp-1 site contained within the Bcl-2 promoter (59). Moreover, it has recently been shown that the survival function of Bcl-2 can be downregulated by phosphorylation within the "loop domain" of the protein (73). E_2 has been shown to prevent the inactivating phosphorylation of this protein by JNK, thereby enhancing breast cancer cell survival (64). Thus the activity and concentrations of this protein are modified by discrete cellular pools of ERs; this allows both rapid and prolonged regulation of this important protein. Another example is the HSP27. Along with other family members, this protein is known to associate with ERs, especially in breast cancer (87). The HSP27 gene is an acknowledged target for nuclear ER transcriptional upregulation (60). Recently, it has been shown that the modulation of HSP27 phosphorylation occurs in response to E_2 acting at membrane ER and that this is critical to the actions of E_2 in ECs (65). Again, the membrane and nuclear pools of ERs have different but complementary actions to regulate the short and longer term cell biological consequences of HSP27 function.

Mechanisms of signaling from the membrane. How does the membrane ER signal? If we assume that the structure of this protein is very similar to the nuclear receptor protein, there does not appear to be a catalytic or kinase domain present. However, E_2 can activate a variety of signaling events, many of which functionally link to activation of G-protein-effected pathways (27, 35). For instance, E_2 rapidly activates membrane adenylate cyclase (2, 57) (often a $G_s\alpha$ function), whereas IP_3 generation and intracellular calcium increases are noted in a variety of cell types (34) (often a $G_q\alpha$ or $G\beta\gamma$ function). Direct evidence that ERs can activate several G protein α-subunits and the resulting signaling comes from CHO cell membranes expressing either ER-α or ER-β (63). Here, IP_3 and cAMP are rapidly

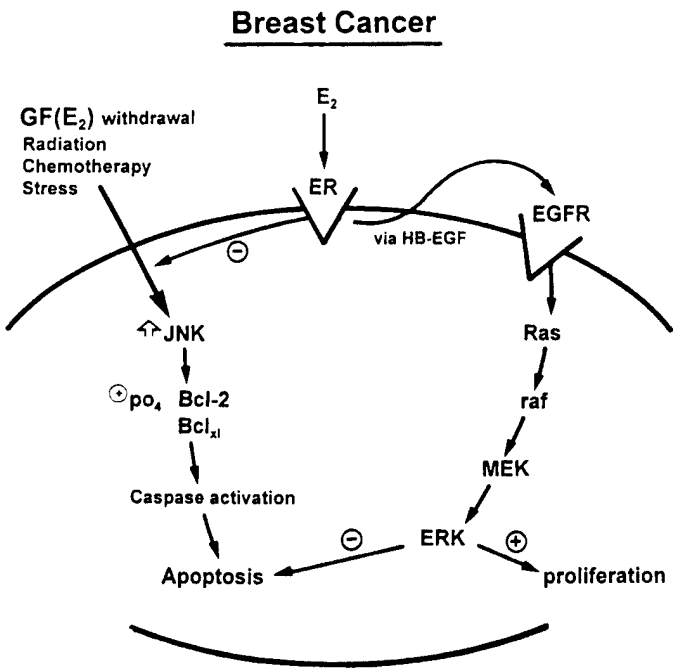

Fig. 2. Schema of E_2 signaling from the membrane in breast cancer. The putative membrane ER activates extracellular-regulated kinase (ERK), via HB-EGF and EGF receptor (EGFR) transactivation, which then signals to cell proliferation. This same pathway contributes to cell survival. The membrane ER also inhibits stress-induced c-Jun NH_2-terminal kinase (JNK) activation, importantly contributing to anti-apoptosis. GF, growth factor; MEK, mitogen-activated protein kinase kinase.

generated in response to E_2, associated with the activation of G_s and $G_q\alpha$. Evidence of direct G protein activation in cells expressing endogenous ER, however, has not yet been shown. Because both G proteins and ERs exist in caveolae, it is likely that an interaction may take place within this membrane domain. Alternatively, it has recently been reported, at least in MCF-7 breast cancer cells expressing endogenous ER, that E_2 can activate a membrane orphan GPCR, GPR30 (16). This was reported to lead to the activation of ERK. Curiously, these events were reported to occur independently of ER, through an undefined mechanism. Many GPCRs have been shown to activate ERK in some cell types through the generation of heparin-binding EGF and the subsequent activation and signaling by the EGF receptor (61). This may contribute to the ability of E_2 to activate ERK, especially in MCF-7 cells (16), and would provide an additional cross-talk mechanism for the observed interdependence of ER and EGF receptor in modulating uterine and breast cancer cell biology (12).

E_2-ER may also utilize other growth factor receptors for signal transduction. In cells expressing cotransfected ER and the insulin-like growth factor (IGF)-1 receptor, E_2 causes the phosphorylation of IGF-1 receptor and enhanced activation of ERK. The two receptors were found to physically associate in this model, as well (24). These interactions may be particularly relevant to breast cancer, since E_2 has been shown to augment the ability of IGF-1 to induce cell proliferation (13), and this may be mediated through insulin receptor substrate-1 upregulation (33). However, in nude mice, MCF-7 cells proliferated despite IGF-1 receptor blockade (3). Also, IGF-1 may phosphorylate and thus activate the nuclear ER to induce transcription (24). There is also evidence that, in autocrine fashion, E_2 can upregulate PC-cell-derived growth factor production from MCF-7 cells (37), although this may result from nuclear ER action. This growth factor had a part in mediating the ability of E_2 to stimulate DNA synthesis in these cells. E_2 may facilitate sex hormone binding globulin signaling from a putative sex hormone binding globulin membrane receptor in prostate cells by an undefined interaction (49). Finally, when both estrogen and androgen receptors are expressed in the same cell, a complex of signaling molecules including both receptors results from ligation by either estrogen or androgen (46). This may serve the purpose of augmenting signal transduction, beyond that which can be activated in response to either sex steroid alone (45).

The importance of the membrane ER might be intuited from recent discoveries in plants. *Arabidopsis* and other flowering plants produce brassinosteroids, compounds that regulate growth and fertility, and share the basic four phenolic ring structure with E_2. There are no nuclear receptors that have been discovered for these plant steroids. However, a transmembrane receptor tyrosine kinase has been found to mediate the binding and signaling of brassinosteroids to cell biology (83). Thus steroid action at the plasma membrane is an ancient and highly conserved function, suggesting its great importance.

FUTURE RESEARCH DIRECTIONS

It is now appreciated that there are multiple pools of endogenous ER expressed in a wide variety of target cells for E_2 action. In fact, an important direction for ER research will be to define the potential roles of the poorly understood cytosolic- localized ER. As an anti-apoptotic factor, cytosolic E_2-ER complexes may locally regulate mitochondrial membrane potential, In support of this idea, it has been shown that E_2 can modulate mitochondrial enzymes (88). It will also be necessary to continue to define rapid, nongenomic actions of E_2 at the membrane receptor, establishing new signal transduction pathways for this receptor, and to learn their impact on cell biology (Fig. 2).

The complementary functions of both membrane and nuclear ERs will best be defined when specific reagents become available to activate or antagonize one receptor but not the other. We also need to understand the unique and overlapping functions of E_2 mediated through ER-α and ER-β, particularly defining the results of heterodimerization in cells expressing both receptors. This is likely to be relevant to both the nongenomic and genomic actions of the sex steroid. Details of how the nuclear receptor organizes the transcriptional apparatus to induce genes and how E_2/ER modulates histone and chromatin will be (and currently is) a topic of much investigation. Equally important is the need to know the structure of the membrane receptor, to understand how it translocates to discrete domains within the membrane, and to understand how it precisely induces signal transduction. It is hoped that by modulating the function of discrete cellular pools of ER with specific agonists/antagonists, we might be able to avoid the unwanted effects of this sex steroid (venous thrombosis, breast cancer promotion, and so forth) but accrue the desirable cardiovascular, bone, and perhaps central nervous system actions of estradiol.

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ERs Associate with and Regulate the Production of Caveolin: Implications for Signaling and Cellular Actions

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Recent evidence supports the existence of a plasma membrane ER. In many cells, E2 activates signal transduction and cell proliferation, but the steroid inhibits signaling and growth in other cells. These effects may be related to interactions of ER with signal-modulating proteins in the membrane. It is also unclear how ER moves to the membrane. Here, we demonstrate ER in purified vesicles from endothelial cell plasma membranes and colocalization of ERα with the caveolae structural coat protein, caveolin-1. In human vascular smooth muscle or MCF-7 (human breast cancer) cell membranes, coimmunoprecipitation shows that ER associates with caveolin-1 and -2. Importantly, E2 rapidly and differentially stimulates ER-caveolin association in vascular smooth muscle cells but inhibits association in MCF-7 cells. E2 also stimulates caveolin-1 and -2 protein synthesis and activates a caveolin-1 promoter/luciferase reporter in smooth muscle cells. However, the steroid inhibits caveolin synthesis in MCF-7 cells. To determine a function for caveolin-ER interaction, we expressed caveolin-1 in MCF-7 cells. This stimulated ER translocation to the plasma membrane and also inhibited E2-induced ERK (MAPK) activation. Both functions required the caveolin-1 scaffolding domain. Depending upon the target cell, membrane ERs differentially associate with caveolin, and E2 differentially modulates the synthesis of this signaling-inhibitory scaffold protein. This may explain the discordant signaling and actions of E2 in various cell types. In addition, caveolin-1 is capable of facilitating ER translocation to the membrane. (*Molecular Endocrinology* 16: 100–115, 2002)

STEROID HORMONES, INCLUDING E2, act by binding nuclear receptors, which then transactivate target genes (1). There is also emerging evidence that E2 has rapid, nongenomic actions (2–4), often originating from the cell membrane. The latter findings are in concert with the identification of plasma membrane ERs that signal when activated by E2 binding (5–7). When ERα or ERβ is expressed in Chinese hamster ovary (CHO) cells, multiple signaling pathways are rapidly activated by E2 despite only 3% of total ERs residing in the cell membrane (6). These same pathways are rapidly activated by E2 in a variety of target cells expressing endogenous ER, and some have been linked to important cellular actions of the steroid (8–10).

Although the plasma membrane ER has not been physically isolated and sequenced, it appears to be very similar to the nuclear protein. Both membrane and nuclear receptors can originate from a single transcript (6), and membrane ER can be identified by antibodies raised against various epitopes of the nuclear receptor (11). The process whereby the ER localizes to the membrane is not known. Also, examination of the nuclear ER sequence does not identify a motif that is homologous to a kinase or catalytic domain of growth factor receptors, binding proteins that are typically inserted into the plasma membrane. Thus, it is not clear how ER enacts signal transduction, but this probably occurs through physical interactions with proteins that functionally modulate signaling. As a mitogen, E2 activates signaling in some target cells (2, 3, 7) but also inhibits signaling and proliferation induced by vascular growth factors in other cells (12, 13). These dichotomous findings illustrate an important but unknown mechanism whereby cytokines can act either in a positive or negative fashion, depending upon the cellular context.

Signaling cascades are activated when a growth factor binds its transmembrane receptor, causing the translocation of proximal signaling molecules to the plasma membrane (14). This often results in the localization of these molecules to subdomains within the membrane bilayer, including rafts and caveolae. Caveolae are ω-shaped, invaginated microstructures that are found in most mammalian cell types and communicate with the cell surface while residing within the membrane (15). They function in vesicular transport, endocytosis, and transcytosis (16), but also play a role in signal transduction (17). Caveolae are predominantly structurally composed of a family of proteins, known as caveolins. Caveolin 1 and 2 are found in

Abbreviations: CHO, Chinese hamster ovary; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; JNK, c-Jun N-terminal kinase; MEK, MAPK kinase; 5′NT, 5′-nucleotidase; NTF2, nuclear transport factor 2; VSMC, vascular smooth muscle cells.

many cells expressing caveolae (18), whereas caveolin 3 is restricted to muscle cells (19). Growth factor receptors are enriched within the caveolae (20) where they bind to caveolin proteins (17) and form complexes with signaling molecules localized to this structure (21). Caveolin-1 contains a cytosolic, N-terminal juxtamembrane domain (scaffolding domain), which binds to signaling molecules and inhibits their usual activation after growth factor ligation of receptors (17, 22). Thus, within the caveolae, an ER interaction with other signaling molecules may be important for the propagation of signal transduction. In the studies reported here, we examined E2-ER interactions with caveolin proteins and the implications for the rapid, nongenomic effects of estrogen.

RESULTS

Endogenous ERα Localizes to Caveolae in the Plasma Membrane

EC were selected because they have high concentrations of endogenous caveolae in the membrane and also express membrane ER (23). To determine the ERα localization within plasma membranes in whole cells, we examined cultured rat lung endothelial cells for possible colocalization of ERα with caveolin-1 by confocal Immunofluorescence microscopy. As shown in Fig. 1A, there is significant colocalization of ERα (*red*) with caveolin-1 (*green*). *Yellow* labeling indicates an extensive but not complete colocalization of the two proteins. This is especially evident in a more three-dimensional depiction of the whole cell (*top*). Thus, by these techniques, we detect the presence of ERα at the cell surface, extensively colocalized with caveolin-1.

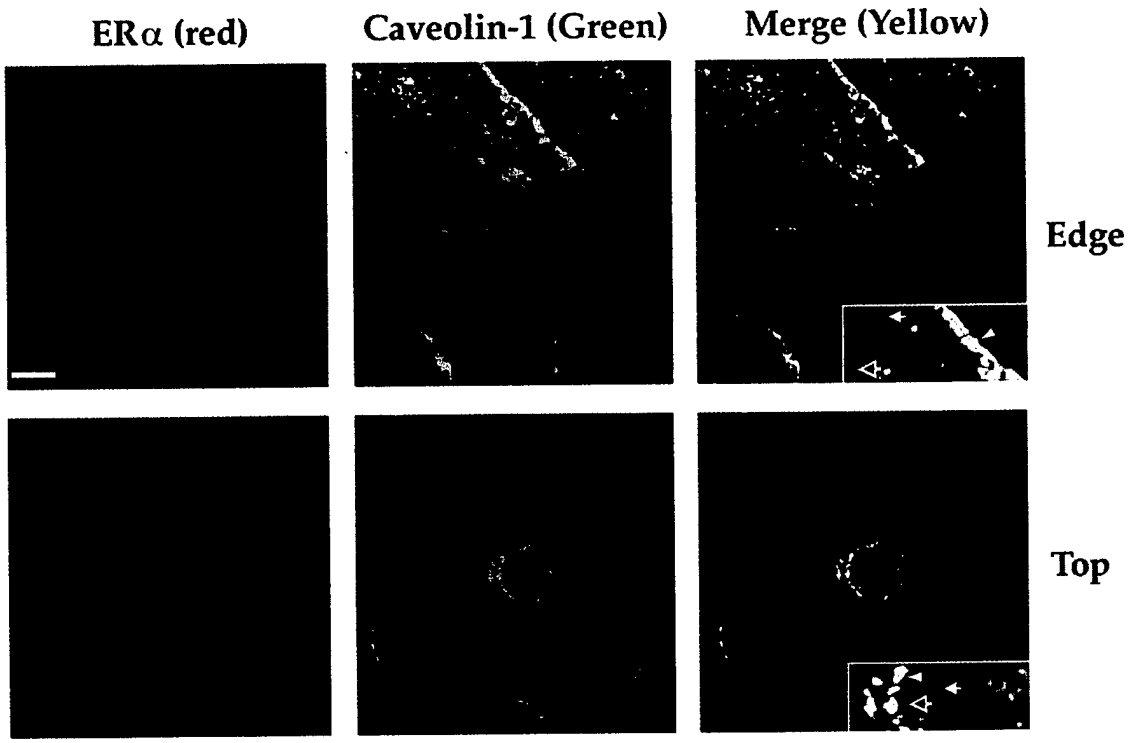
We then carried out immunoblots of cell fractions from the endothelial cells. Rat lung tissue was subfractionated to isolate first the luminal endothelial cell plasma membranes (P) and then their caveolae (V) (15, 24, 25). As shown in Fig. 1B, immunoblot analysis of silica-coated plasma membrane (P) displayed ample enrichment for plasma membrane markers such as 5′ nucleotidase (5′NT, a glycosyl phosphatidyl inositol-anchored protein) and caveolin-1 (caveolae coat protein) relative to the starting whole lung homogenate (H). ERα is easily detected in P, but unlike 5′NT and caveolin-1, it is not greatly enriched in this fraction relative to the whole lung homogenate. Molecular mass markers (not shown) indicate that ER migrates at approximately 62 kDa. Because ERα is mostly found located in the nuclear membrane, we tested for markers of intracellular organelles including nuclear membrane proteins. P was markedly depleted of the nuclear membrane proteins, transportin and nuclear transport factor 2 (NTF2), as well as the endosomal/Golgi marker β-Cop. Thus, ERα appears to be present at the cell surface using these fractionation techniques.

The caveolae attached on the cytoplasmic side of the membranes opposite to the silica coating were stripped by shearing and then were isolated by sucrose density centrifugation to yield a low buoyant density fraction of intact caveolar vesicles (V). This was well separated from the membranes stripped of the caveolae (P-V). As show in Fig. 1B, ERα is enriched in V relative to P. It is also detected in P-V, but to a lesser extent than in V. This result is consistent with the confocal localization, where ER colocalized extensively but not completely with caveolin-1. Note that the nuclear membrane proteins, transportin and NTF2, are not detected in V. As we previously reported (15, 24, 25), V was enriched in caveolin-1 but not 5′NT. Little caveolin-1 signal remained in P-V.

We also took advantage of another technique (albeit not our preferred method) that does not use silica coating for caveolae isolation (25). Here, the homogenized lung is subjected to percoll gradient centrifugation to yield a plasma membrane-rich fraction (PM) that contains the plasma membrane markers 5′NT and caveolin-1 but also, unfortunately, β-Cop and one of the two nuclear membrane proteins (transportin, but not NTF2). Sonication of PM followed by flotation via sucrose density centrifugation yielded a low buoyant density fraction (AC) quite enriched in caveolin-1. 5′NT, transportin, and β-Cop, are readily detected in AC, whereas NTF2 is only found in the homogenate (H) with little to no signal elsewhere. We subjected AC to further subfractionation to isolate the caveolae more selectively by immunoaffinity separation. 5′NT, transportin, and β-Cop are all detected in the unbound fraction (U) with little to no signal in the immunoisolated, caveolin-coated caveolae bound to the magnetic beads (B). Both ERα and caveolin-1 are found enriched in the immunoisolated, caveolin-coated caveolae bound to the beads (B). Thus, ERα and caveolin-1 are in the same vesicles. Past work shows that B and V appear equivalent in molecular composition and both methods agree here. Thus, ERα is contained within low-density, caveolin-coated plasmalemmal vesicles, namely caveolae.

Caveolin Proteins Differentially Associate with Membrane ERs in MCF-7 and Vascular Smooth Muscle Cells (VSMC)

Caveolin proteins constitute an important structural component of caveolae but are also found to a smaller extent in noncaveolar fractions of the membrane. We next determined whether ERα and caveolin proteins could associate in the membrane. This is potentially important to begin to understand the organization of signaling molecules within this membrane domain. We turned to cell systems in which we have previously characterized E2 signaling through the membrane and the attendant effects on cell biology (9, 12). Thus, MCF-7 and VSMC serve as potential models by which to investigate how E2 can differentially stimulate or inhibit signaling. We first immunoprecipitated ERα in



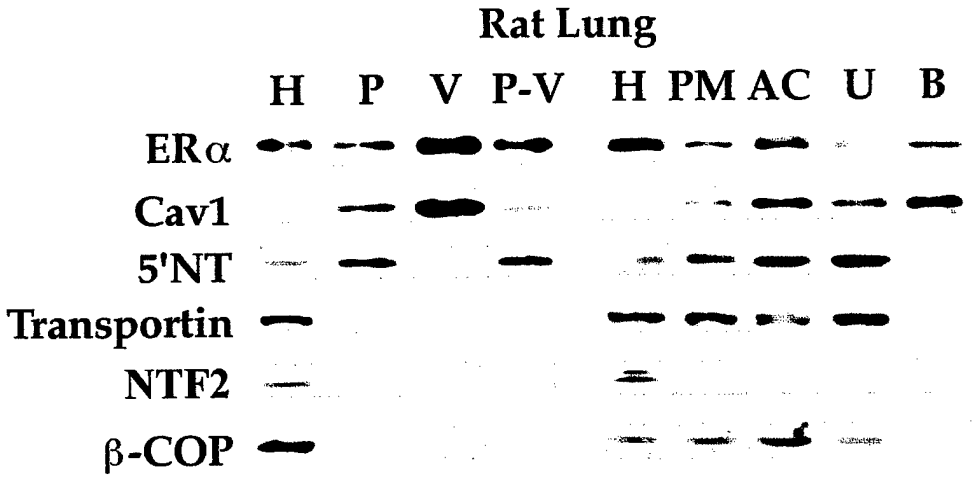


Fig. 1. ERα Colocalizes with Caveolin-1 in the Plasma Membrane of Endothelial Cells

A, Immunofluorescence microscopy shows significant ERα and caveolin-1 colocalization. Antibodies to ERα and caveolin-1 were used to determine colocalization between the two proteins in rat lung endothelial cells, followed by the appropriate reporter antibodies (goat antirabbit-Texas Red (*red*, ERα) and goat antimouse-Bodipy FL (*green*, cav-1). Merge is an overlay of the ERα (*red*) and cav1 (*green*) signal. Edge is the flattened leading edge of the cell, whereas Top refers to the top crest of the cell. *Arrowhead* indicates ERα and cav-1 colocalization, *white arrow* indicates ERα localization without colocalization with cav-1, and *black arrow* indicates caveolin-1 without colocalization with ERα. The *insets* are 3× magnification. B, ERα is contained within caveolae (V & B) fraction of EC membranes. The *left panel* shows the colloidal silica-coating technique to subfractionate rat lung into total homogenate (H), silica-coated luminal endothelial cell plasma membranes (P), caveolae (V), and the repelleted silica-coated membranes stripped of caveolae (P-V). The *right panel* shows the immunoisolation technique for total lung homogenate (H), plasma membrane-rich fraction (PM) by percoll gradient, and caveolin-rich fraction (AC) by sucrose gradient centrifugation. Immunoisolated caveolae were recovered using antibodies to caveolin-1, which fractionates material bound (B) to the beads (caveolae) or unbound (U) in the supernatant. Antibody to the C terminus of ERα was used, along with other appropriate antibodies to the indicated proteins (see *Results*), for Western blot of each fraction (2 μg/lane). The experiments were repeated three times.

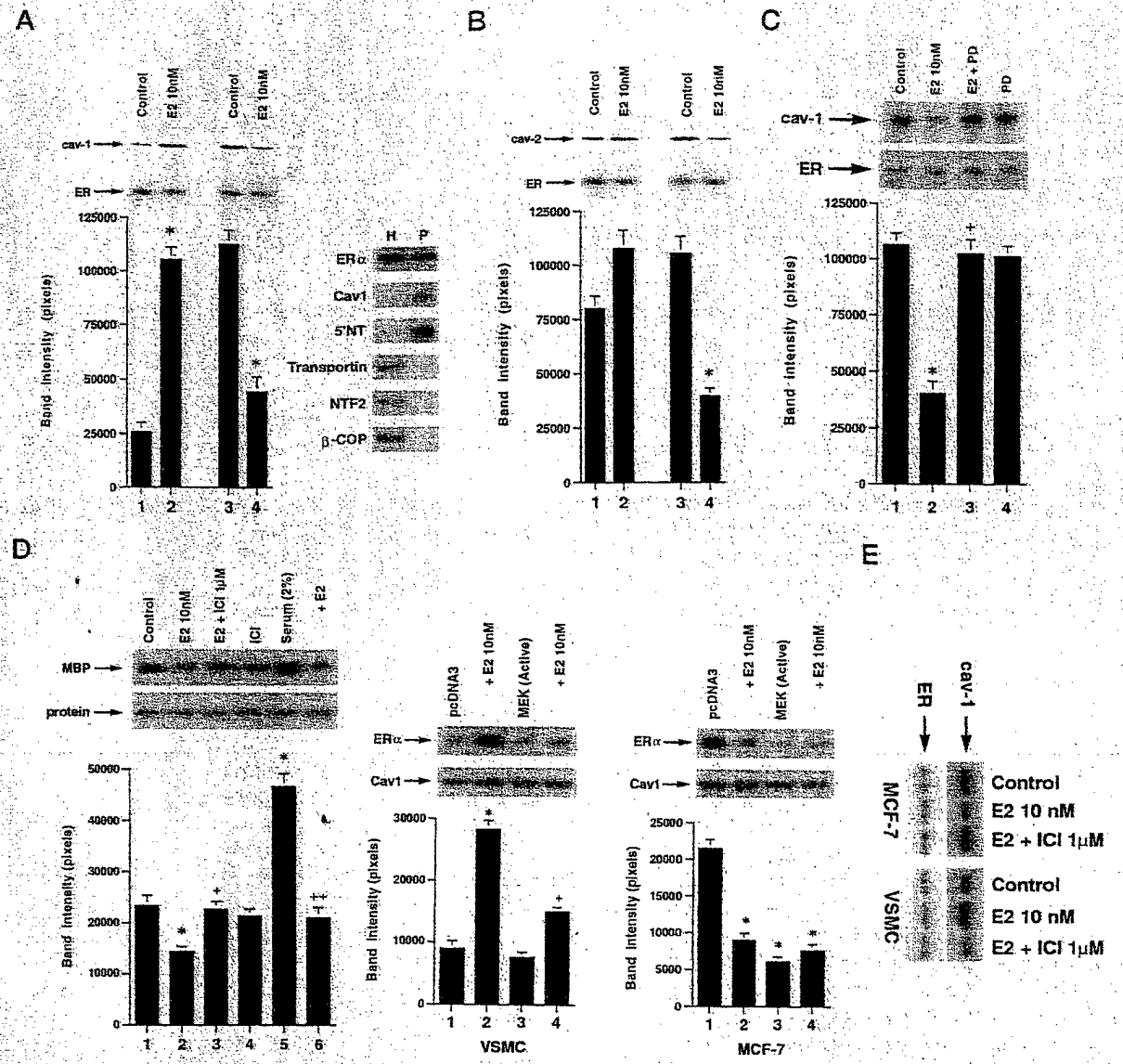


Fig. 2. Caveolin-ERα Association Is Regulated by E2 and ERK

A, *Top*, E2 differentially modulates the association of caveolin-1 and ERα in membranes from VSMC (lanes 1 and 2) and MCF-7 (lanes 3 and 4). Cells were treated, or not, with 10 nM E2 for 30 min, after which the cells were lysed and membranes were prepared as described in *Materials and Methods*. ER was immunoprecipitated, followed by Western blot for caveolin-1. ER protein immunoprecipitated from each condition is also shown. *Bottom*, Immunoblot detection of ERα, caveolin-1, 5′NT (NT), transportin (Trans), NTF2, and β-Cop from whole MCF-7 cell homogenate (H) or sucrose gradient-fractionated plasma membranes (P). B, Differential association of caveolin-2 with ERα. C, E2 inhibition of ER/caveolin-1 association in MCF-7 cells is ERK dependent. Cells were incubated for 30 min with 10 nM E2 ± PD98059, a soluble MEK inhibitor. The *bar graphs* reflect three experiments combined. *, $P < 0.05$ for control *vs.* E2, +$P < 0.05$ for E2 *vs.* E2 + PD98059 by ANOVA plus Scheffe's test. D, *Left*, E2 inhibits ERK activity in VSMC. Cells were incubated with or without 10 nM E2 for 8 min, sometimes in the presence of 2% serum, and ERK activity was determined using the substrate protein myelin basic protein. ERK protein as loading controls is seen *below* each condition, and the *bar graph* represents three combined experiments.*, $P < 0.05$ for control *vs.* E2 or serum; +, $P < 0.05$ for E2 *vs.* E2 + ICI182,780; ++, $P < 0.05$ for serum *vs.* E2 + serum. *Right*, Expression of active MEK-1 differentially modulates ER-caveolin-1 association. MEK-1 expression inhibits E2-induced ER-caveolin-1 association in VSMC, and (independently of E2) inhibits basal ER-caveolin-1 association in MCF-7. The cell lysates were immunoprecipitated for caveolin-1 and then immunoblotted for ERα. Equal amounts of protein and immunoprecipitated caveolin are shown *above* the *bar graph*. The data are from three experiments combined; *, $P < 0.05$ for expressed pcDNA3 control *vs.* condition; +, $P < 0.05$ for pcDNA3 + E2 *vs.* MEK + E2 in VSMC. E, E2 modulation of ER-caveolin association occurs through binding to ER. Cells were incubated with E2 ± ICI 182,780, a specific ER antagonist, and three studies were combined.

the membranes of both VSMC and MCF-7 cells, followed by blotting against caveolins, and found that ERα can associate with either caveolin-1 or -2 (Fig. 2). Similar results were found by immunoprecipitating caveolin, followed by ER blotting (data not shown). Interestingly, ligation of ER with E2 for 30 min strongly alters the above association, and this occurs differentially in the two cell types. In VSMC, there was relatively little association of ER and caveolin-1 in basal cells, but in response to E2, the association increased 3-fold (Fig. 2A, *top*, lanes 1 and 2). However, in the basal MCF-7 cells, there was a relatively strong association of these two proteins in the membrane, but E2 addition caused a 67% down-regulation of this interaction (Fig. 2A, *top*, lanes 3 and 4). Similar results were found for caveolin-2, although the E2 stimulation of ER-caveolin-2 association was not as strong (only 50% increased) in the VSMC (Fig. 2B). To support the purity of the sucrose gradient-isolated plasma membranes, immunoblots for ER and caveolin-1, 5′NT (integral membrane protein), transportin, and NTF2 (nuclear proteins), and the endosomal/Golgi marker β-Cop were carried out. As seen in Fig. 2A (*bottom*), 5′NT and caveolin-1 were enriched in the plasma membrane fraction (P). However, the other three proteins were not found, despite their presence in whole cell homogenates. ERα was detected in both samples, as expected.

We then investigated whether the ability of E2 to activate ERK (MAPK) influenced steroid association with caveolin. As shown in Fig. 2C, abolishing ERK activation with the soluble MAPK kinase (MEK) inhibitor, PD98059, strongly prevented the inhibition of ER-caveolin-1 association in MCF-7 cells. We previously showed that E2 rapidly activates ERK in this human breast cancer cell line, and PD98059 blocks this action (9). These data suggest that downstream signal transduction through ERK feeds back to inhibit the association of ER and caveolin-1 proteins in the membrane after E2 treatment. To corroborate a role for ERK, we transiently transfected/expressed a constituitively active MEK-1 into the MCF-7 cells and determined ER and caveolin-1 association. This kinase construct is well known to directly stimulate ERK activity. We found that independent of exposing the cells to E2, active MEK could also stimulate the dissociation of the two proteins in the membrane (Fig. 2D, *right*).

We then assessed the situation in VSMC. First, we determined the ability of E2 to affect ERK activity in VSMC (Fig. 2D, *left*). The low basal ERK activity was inhibited 44% by 10 nM E2, and the specific ER antagonist, ICI 182,780, prevented this. Furthermore, serum-induced ERK activity was almost completely blocked by E2. When we expressed an activated MEK construct in the VSMC, and incubated the cells with 10 nM E2, the augmented ER-caveolin-1 association seen with E2 alone was significantly prevented (Fig. 2D, *right*). Thus, the ability of E2 to differentially modulate ER-caveolin-1 association in the two cell types is consistent with its differential effects on ERK activity. This, in turn, is consistent with the known differential effects of E2 on cell proliferation, positive in MCF-7 and negative in VSMC, where proliferation/antiproliferation is related to the appropriate modulation of ERK activity (7, 12). We also determined that ICI 182,780 blocked the association-modulating actions of E2 in both cell types (Fig. 2E), consistent with ICI 182,780 effects on E2-modulated ERK activity. Overall, this indicates that E2 specifically acts through ER to signal, and thus influence, the association of ER-caveolin.

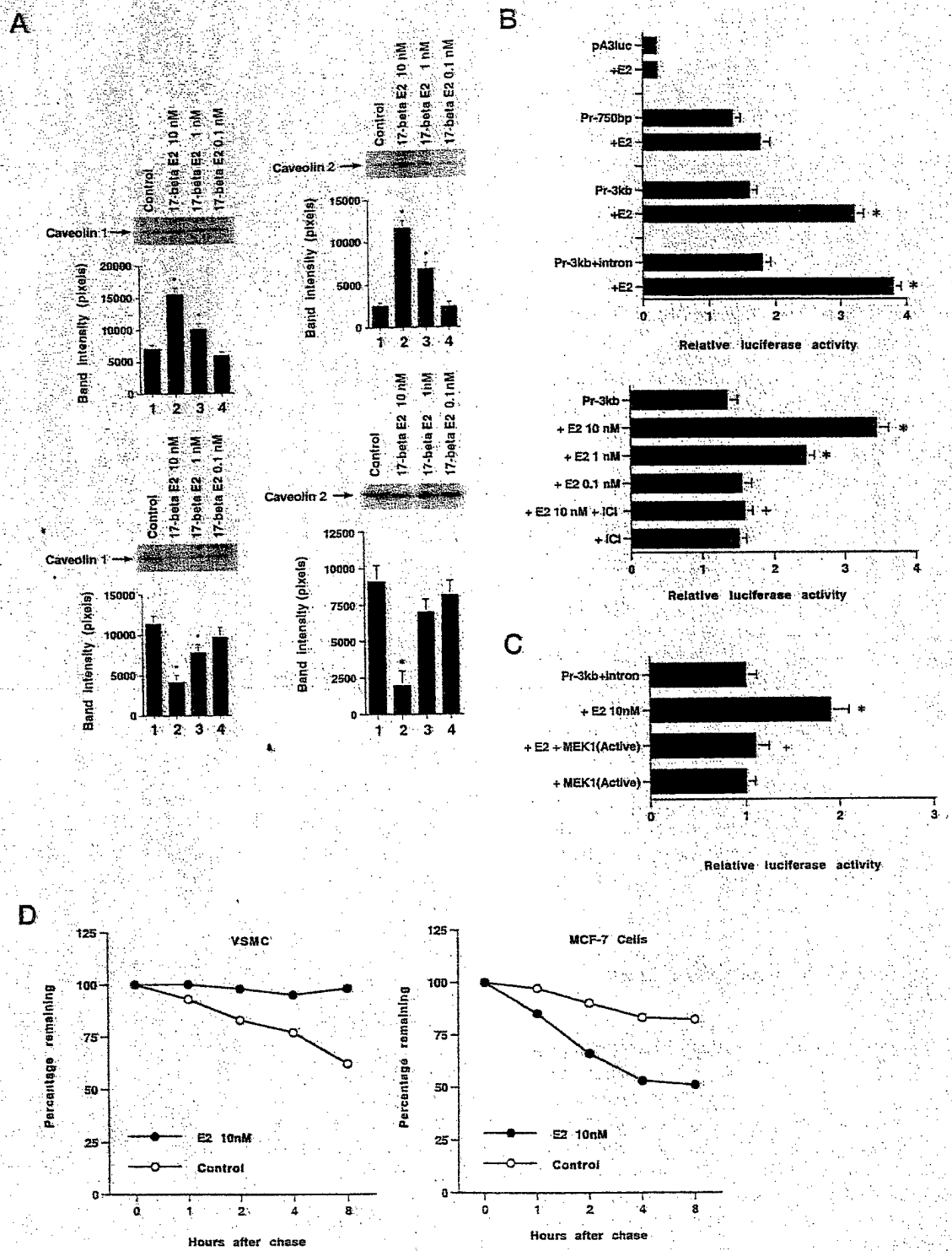
E2 Differentially Modulates Caveolin Production in MCF-7 and VSMC

E2 could modulate the chronic interactions of ER-caveolin in part by affecting caveolin production. This would likely occur through the actions of the nuclear receptor. We therefore determined whether E2 could regulate caveolin protein synthesis. In VSMC, E2 stimulated the production of newly synthesized caveolin-1 and -2 proteins after 8 h incubation in a dose-responsive manner (Fig. 3A, *upper*). These effects were significant at physiologically relevant concentrations of 1 and 10 nM E2. In contrast, E2 significantly inhibited caveolin-1 and -2 new protein production in MCF-7 cells (Fig. 3A, *lower*). These findings could have important implications for the ability of E2 to signal, since caveolin often serves as an inhibitory scaffold protein (17, 22).

As postulated, the modulation of caveolin protein synthesis demonstrated here may result from the ability of the nuclear ER to regulate the transcription of caveolin. We therefore examined the effect of E2 on caveolin-1 promoter/luciferase reporters, expressed in VSMC. Compared with the empty vector, pA3-Luc, basal reporter activity increased from the expression of the −750-bp Cav-1/Luc construct (26), but this reporter was not estrogen responsive (Fig. 3B, *top*). In

Fig. 3. E2 Modulates the Expression of Caveolin-1

A, E2 stimulates the synthesis of caveolin-1 (*upper left*) and caveolin-2 (*upper right*) in VSMC, while inhibiting caveolin-1 (*lower left*) or caveolin-2 (*lower right*) protein synthesis in MCF-7 cells. Cells were first labeled with ^{35}S-methionine and then incubated with or without E2 for 8 h. Caveolin proteins were immunoprecipitated and resolved by PAGE. *Bar graph* data are combined from three experiments. *, $P < 0.05$ for control *vs.* E2. B, *Top*, E2 stimulates the activity of caveolin-1 promoter/luciferase reporters. pA3-Luc (control), and Pr-750bp, Pr-3 kb, and Pr-3 kbandInt 1/pA3 Luc, which contain various lengths of the caveolin-1 promoter. were transfected into VSMC, as described in *Materials and Methods*. The cells were then incubated with 10 nM E2 for 8 h. *Bottom*, Dose responsiveness of the Pr-3 kb caveolin-1 promoter/luciferase reporter activity to E2. Experiments were similarly carried out. Concentrations of E2 are expressed as molar. Experiments were carried out four times, each condition in triplicate during each experiment, and the data were combined. *, $P < 0.05$ for control plasmid expression (pA3-Luc or Pr-3 kb) *vs.* E2-treated cells;



+, $P < 0.05$ for E2 *vs.* E2 + ICI 182,780 (ER antagonist). C, Expression of activated MEK-1 prevents E2-induced transactivation of the caveolin-1 promoter/luciferase reporter. VSMC were first transfected to express the −3 kb + intron reporter ± MEK-1, recovered overnight, and then incubated with 10 nM E2. A representative study with triplicate determinations per condition, repeated twice, is shown. *, $P < 0.05$ for control plasmid expression (Pr-3 kb plus intron) *vs.* E2-treated cells; +, $P < 0.05$ for E2 *vs.* E2+MEK-1. D, E2 differentially modulates the stability of the newly synthesized caveolin-1 protein in cell-specific fashion. After labeling with ^{35}S-methionine, followed by unlabeled methionine, caveolin-1 was immunoprecipitated from lysed VSMC (*left*) or MCF-7 (*right*) over an 8-h time course, in cells exposed or not exposed to 10 nM E2. Caveolin-1 proteins were immunoprecipitated, proteins were normalized before gel loading, and caveolin-1 was resolved by PAGE, followed by densitometry. The data are from a single representative experiment, repeated twice.

contrast, the −3 kb, and −3 kb and intron Cav-1/luciferase reporters were significantly and equally responsive to E2. Further, E2 at 1 and 10 nM stimulated the activity of this reporter (Fig. 3B, *bottom*), and ICI 182,780, the ER-specific antagonist, strongly prevented this action of 10 nM E2. In scanning the 3 kb of promoter sequence, there is no palindromic estrogen response element, but there are numerous transcription factor-binding sites that could potentially mediate the action of E2. These data suggest that the E2-induced synthesis of caveolin-1 likely results from the stimulation of transcription.

Does the ability of E2 to inhibit ERK (see Fig. 2D, *left*) lead to the up-regulation of the reporter fusion gene activity in theses cells? We found that expression of active MEK-1 significantly inhibited the stimulatory effect of E2 (Fig. 3C). These results support overall the ideas that E2 inhibition of ERK stimulates ER-caveolin association (Fig. 2D, *right*) and the transcriptional transactivation of caveolin-1 in the VSMC.

We then asked whether E2 might also modulate the stability of the caveolin-1 protein. To examine this, we carried out pulse-chase labeling studies in both MCF-7 and VSMC, in the presence and absence of E2 (Fig. 3D). In VSMC, there was a 35% degradation of newly formed caveolin-1 protein over an 8-h period in the absence of E2. In contrast, the steroid completely prevented this degradation. Thus, the increased levels of VSMC caveolin-1 protein in the presence of E2 reflect both the stimulation of transcription/synthesis, as well as the inhibition of protein loss. In MCF-7 cells, the loss of newly produced, labeled caveolin-1 over 8 h was only 13% in the absence of E2 (Fig. 3D, *right*). However, the sex steroid accelerated caveolin degradation, yielding a 45% decrease during this same time period. Therefore, E2 inhibits the concentration of caveolin-1 protein in these cells through several mechanisms.

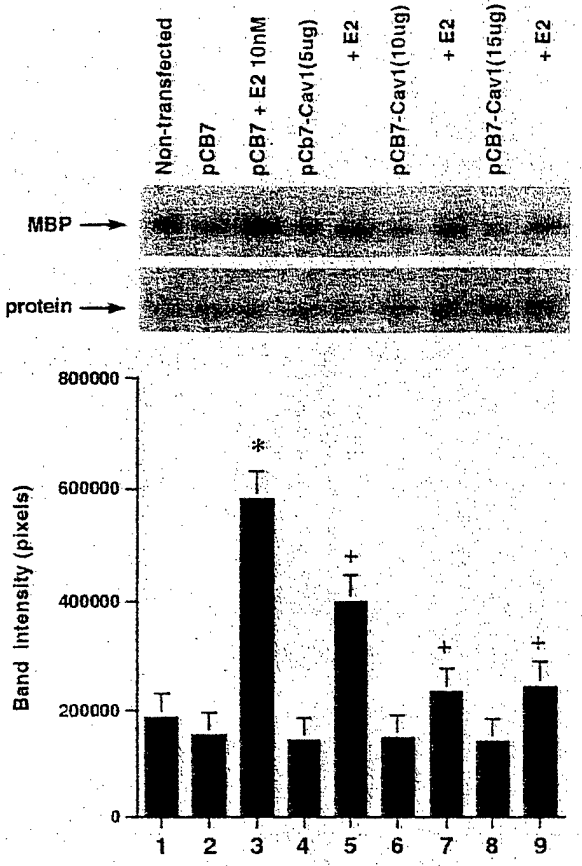


Fig. 4. Concentration-Related Inhibition by Caveolin-1 of E2 Activation of ERK in MCF-7 Cells

Increasing amounts of full-length caveolin-1 cDNA in pCB7 were expressed after transient transfection. The cells were then treated with or without E2 for 8 min and then lysed, and ERK activity was immunoprecipitated for an *in vitro* assay using myelin basic protein as substrate (see *Materials and Methods*). Immunoblot of ERK protein is shown *below* a representative study. Three experiments were combined for the *bar graph*.

Caveolin Expression Inhibits E2-Induced ERK Activity

What might be the role of caveolin-ER interactions in the membrane? In MCF-7 cells, E2 rapidly stimulates signaling to ERK activation, which is necessary for DNA synthesis or cell survival (7, 9). In contrast, E2 inhibits growth factor activation of ERK and the proliferation of VSMC (12). We hypothesized that the differential modulation of caveolin production/association that we have shown here might explain these divergent actions in different cell types.

To support this hypothesis, we transfected different amounts of caveolin-expressing plasmid (pCB7Cav-1) and assessed the ability of E2 to stimulate ERK activity. In MCF-7 cells expressing the empty vector (Fig. 4, lane 2), E2 caused a 3-fold increase in MAPK activity (lane 3). Transfecting increasing quantities of caveolin-1 plasmid led to a concentration-related decrease in the ability of E2 to activate ERK. The maximal 83% inhibition was seen with 10 μg of caveolin-1 plasmid (lanes 6 and 7, compared with lanes 2 and 3). These results directly indicate that caveolin impairs E2 signaling through ERK, consistent with its reported negative signaling function (17, 22). In VSMC, E2 stimulates caveolin production and enhances ER-caveolin association. These findings provide a mechanism for our previous observations that E2 inhibits ERK activation and ERK-mediated, growth factor-induced cell proliferation *in vitro* (12).

Caveolin Expression Facilitates the Translocation of ER to the Membrane

What other ER functions might caveolin modulate? The events that result in ER being localized to the plasma membrane are unclear. One possibility is that ER translocation from cytoplasm to the membrane is facilitated by caveolin-1, because this protein can move from the cytosol, in and out of the

plasma membrane (18). To test this idea, we expressed caveolin-1 and determined the relative ratios of ER in the cytosol and the cell membrane (Fig. 5A). The plasma membranes were found to be free of several nuclear or cytoplasmic proteins, determined by immunoblotting as shown in Fig. 2A. In nontransfected cells or in cells expressing the empty vector, pCB7, more ER resides in the cytoplasm, relative to the cell membrane (lanes 1–4). Caveolin-1 expression caused a clear increase in the localization of ER at the plasma membrane, as well as a decrease in the cytosolic content of this protein (lanes 3 and 4 *vs.* 7 and 8). In the absence of exogenous caveolin expression, incubation of the cells with E2 modestly stimulated the translocation of ER to the membrane with a concurrent diminution in cytoplasmic steroid receptor (lanes 3 and 4 *vs.* 5 and 6). Expression of caveolin plus E2, however, was not additive.

We then compared the ability of wild type and a scaffolding domain-deleted caveolin-1 (Δ 60–100) as to their ability to translocate ER to the membrane (Fig. 5B). It has been established previously that caveolin-1 that lacks this domain is incapable of reaching the cell surface (27). The mutant caveolin-1 was nearly 60% less efficient in reducing the cytoplasmic pool and increasing the membrane localization of ER [lanes 3 and 4, compared with lanes 5 and 6 (wild type), then lanes 7 and 8 (Δ 60–100)]. Equal amounts of caveolin protein were expressed using the two constructs, determined by immunoblot (data not shown). Our findings indicate 1) a novel property of caveolin to promote membrane localization of ER, and 2) that the scaffolding domain of caveolin-1 appears to be important for this effect.

To further support the role of caveolin-1 to facilitate ER localization at the membrane, we examined Caco-2 rat intestinal epithelial cells. These are among the few mammalian cells that lack caveolae and do not produce caveolin-1 protein (28). We confirmed the lack of caveolin-1 by immunoblot studies in the native cells. These cells are reported to produce a small population of ER when confluent, which we confirmed by our binding studies (see below). In the control or pcDNA3 transfected cells, there was virtually no specific binding in the membrane, and a small amount of binding in the nucleus, consistent with a modest expression of endogenous ER detected in the latter location (Fig. 3C). Upon expression of ERα, specific binding was clearly detectable but was somewhat modest at the membrane (Fig. 3 C, *left*). In part, this reflected our transfection efficiency in these cells, which was approximately 20%, determined by using a green fluorescent protein-ERα construct. However, coexpression of caveolin-1 increased the specific binding by labeled E2 at the membrane nearly 70%. Expression of caveolin-1 in the absence of ER was similar to that of control (data not shown). In the nucleus, there was much more specific binding after ER transfection (Fig. 5C, *right*). This likely reflected the predominance of ER at this location, similar to what we have shown in CHO cells (6). However, caveolin expression did not further enhance the binding of E2 at this site, indicating that there was no facilitation of ER moving to this location in this model. The results from the nuclear fraction rule out an effect of caveolin to enhance E2 binding through a mechanism apart from facilitating receptor translocation/number. The differential effects at the two sites also support the lack of perinuclear membranes contaminating our plasma membrane fractions. These findings in a non-overexpression model support our contention that caveolin significantly facilitates ER translocation to the plasma membrane.

Role of the Scaffolding Domain in ER-Caveolin Interactions

If caveolin-1 facilitates ER translocation to the membrane, then it is probably necessary for the two proteins to associate in the cytoplasm. Furthermore, it is not clear whether the scaffolding domain of caveolin-1 is needed for the protein-protein interaction with ER or whether it serves to target the steroid receptor to the membrane. The latter role would be consistent with a known function of the scaffolding domain (17). To examine this, we transfected full-length or scaffolding domain mutant caveolin-1 into MCF-7. We then immunoprecipitated ER from cytosol, followed by blotting for caveolin-1, and also carried this out in reverse order. As seen in Fig. 6A (*upper*), there is a strong association of caveolin-1 with ERα, and the association is the same whether expressing full-length or scaffolding domain-deleted mutant caveolin-1. Upon transfection with either construct, Western blot for caveolin-1 revealed comparable amounts of caveolin-1 protein (*lower bands*). Furthermore, the same amount of endogenous ER protein was expressed across the experimental conditions. We therefore conclude that that these two proteins associate both in cytosolic and membrane compartments of the cell, and the scaffolding domain is not generally required for the physical association of caveolin-1 with endogenous ER.

This led us to propose that the ability of caveolin to inhibit E2/ER signaling to ERK is dependent upon membrane localization of the two molecules. This is an important issue because it is likely that ER interacts in the membrane with components of the signal cascade that activate this MAPK. Based upon the previous experiments (Fig. 5), we suggest that membrane targeting of ER is dependent, in part, upon the scaffolding domain of caveolin-1. We therefore determined whether full-length caveolin-1, but not the scaffolding domain mutant, was capable of inhibiting E2 activation of ERK in MCF-7. Consistent with the overall data, we found this to be the situation, in that only the full- length caveolin protein significantly inhibited E2 activation of ERK (Fig. 6B). Thus,

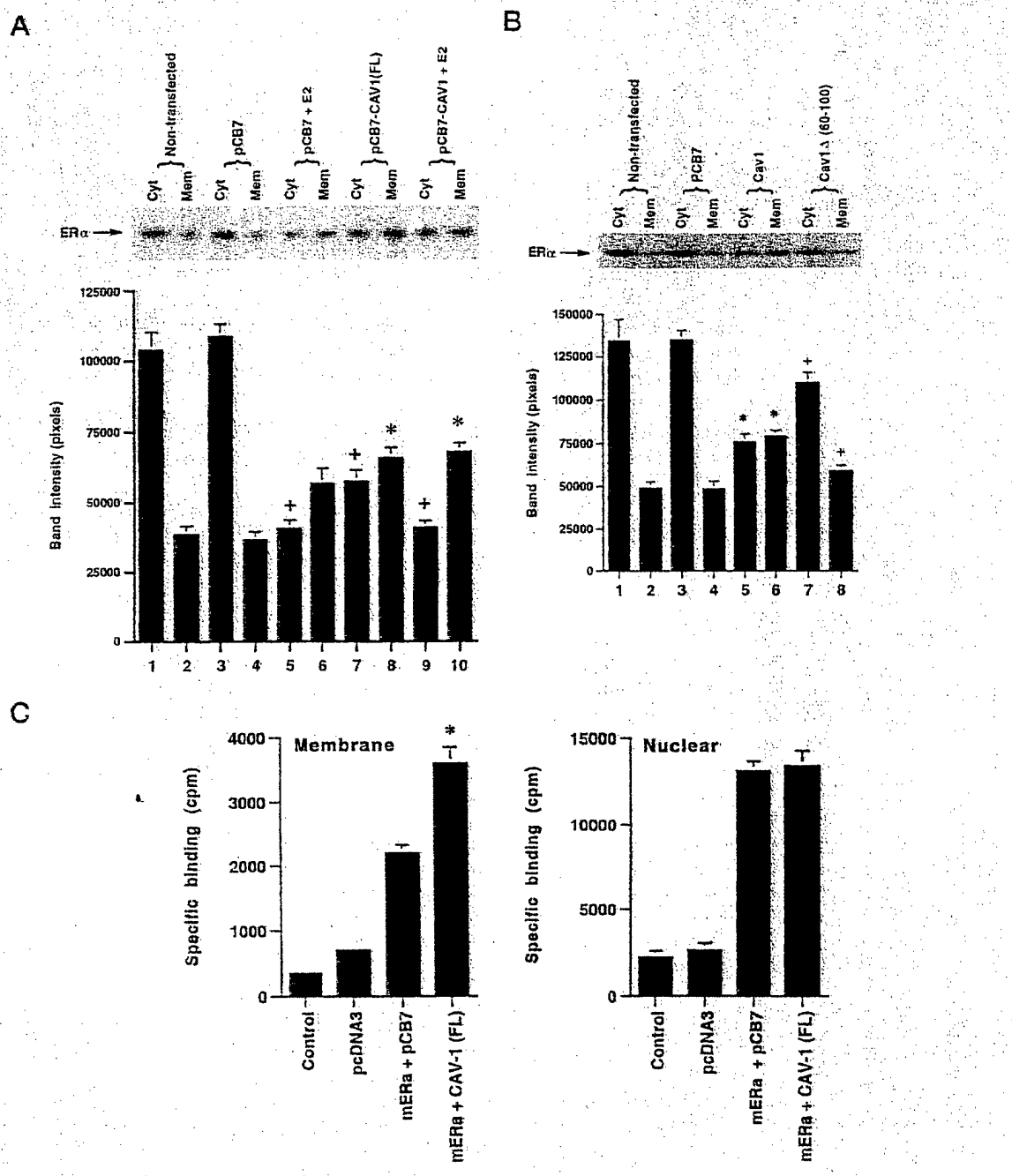


Fig. 5. Caveolin-1 Expression Facilitates ERα Translocation to the Plasma Membrane

A, Expression of caveolin or incubation of MCF-7 with E2 each stimulates translocation of ER from the cytosol to the plasma membrane. Cells were transfected with wild-type caveolin-1 or incubated with E2 without exogenous caveolin, and after ultracentrifugation and percoll gradient isolation, ER localization in cytoplasm and membrane was determined by Western blot. Total protein from cell lysate fractions was determined, and equal aliquots from the conditions were loaded on the gel. A representative study is shown, repeated three times for the *bar graph*. +, $P < 0.05$ for cytoplasmic ER control (lane 3) *vs.* E2-treated cells (lane 5), caveolin-expressing (lane7), or both (lane 9). *, $P < 0.05$ for membrane ER control (lane 4) *vs.* caveolin-expressing cells (lane 8), or caveolin + E2 (lane 10). B. A scaffolding domain-deleted caveolin-1 protein is ineffective in translocating ER to the membrane. MCF-7 were transfected with wild-type or Δ (60–100)cav-1, and ER localization in cytoplasm and membrane was determined by Western blot. *, $P < 0.05$ for cytoplasmic or membrane ER control (lanes 3 and 4) *vs.* wild-type caveolin-expressing cells (lanes 5 and 6, respectively); −, $P < 0.05$ for wild-type caveolin-expressing cells (lanes 5 and 6) *vs.* scaffolding domain-deleted caveolin-expressing cells (lanes 7 and 8). C, Expression of caveolin-1 in Caco-2 cells enhances E2 binding only at the membrane. Caco-2 cells were not transfected (control) or transfected to express ERα without or with cotransfection of caveolin-1. Specific binding of ^{3}H-E2 was determined in membrane (*left*) and nuclear (*right*) fractions. The data are from duplicate determinations/condition, in each of two experiments. *, $P < 0.05$ for ERα-expressing *vs.* cotransfected ERα plus caveolin-1.

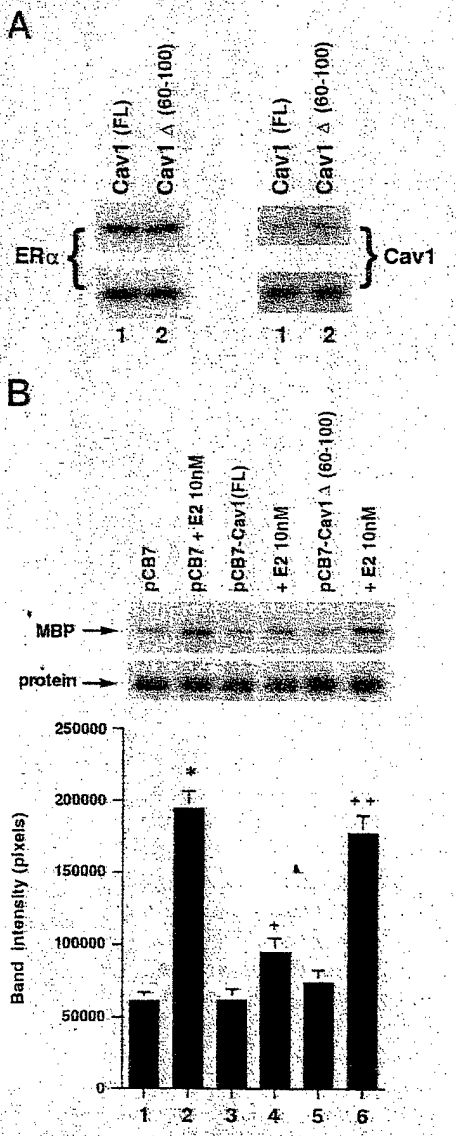


Fig. 6. Full-Length Caveolin-1 Associates with Cytosolic ERα and Is Necessary for Membrane Localization

A, Caveolin-1 and ERα associate in the cytosol of MCF-7 cells. MCF-7 were transiently transfected to express either full-length (lanes 1) or scaffolding domain-deleted (lanes 2) caveolin-1, and then the cytosol fractions were isolated by centrifugation of whole-cell lysates. Either caveolin-1 or ERα was immunoprecipitated, followed by immunoblot for ERα (*left upper*) or caveolin-1 (*right upper*). Western blot for total ERα or cav-1 protein is seen in the *lower* bands. The data are representative of two separate experiments. B, Expression of full-length but not the scaffolding domain mutant caveolin-1 inhibits E2/ER activation of ERK. MCF-7 cells were transfected as above and then ERK activity was determined as described. Data are from three separate experiments combined for the *bar graph*.

the scaffolding domain promotes ER targeting to the membrane, and this also allows the functional interaction with wild-type caveolin-1 that down-regulates E2/ER signaling from this location.

Targeted Expression of ERα E Domain to the Plasma Membrane Activates ERK

To this point, we have demonstrated the localization of ER in the plasma membrane and have presented data to support the proposal that it is the membrane, and not the nuclear ER, that participates to activate ERK. Another approach to this latter, important issue is to target ER to each of the two cell locales and determine the effects of E2. Recently, Kousteni *et al.* (29) showed that E2 acts through the cell membrane ER to prevent etoposide-induced osteoblast cell death. This occurred after E2 activated a signal transduction cascade that ultimately resulted in ERK activation, and this signaling was required for cell survival. These authors also targeted just the E domain of ERα to the plasma membrane and showed that this was sufficient to prevent HeLa cell death. Signaling to ERK was not determined in the targeting model.

We therefore asked whether targeting the E domain to the plasma membrane could result in the ability of E2 to activate ERK. We found that in CHO cells transfected to express this domain at the plasma membrane (E-Mem-ECFP) (29), E2 strongly activated ERK activity (Fig. 7). In contrast, when the E domain was targeted to the nucleus (E-Nuc-ECFP), there was no activation of this MAPK by E2. E2 also could not activate ERK in the native CHO cells, which we previously showed do not express endogenous ER (6). Expression of the membrane-targeted E domain did not result in E2 activating the −3 kb caveolin-1/luciferase reporter (data not shown). This was expected, since in the VSMC, expression of active MEK (and hence ERK) suppressed E2-induced caveolin-1/luciferase reporter activation. Thus, it is likely that, in response to steroid, the E domain portion of the membrane ERα activates the signaling to MAPK activation.

DISCUSSION

The existence of ER in the plasma membrane is now supported by studies from several laboratories (5, 23) in addition to our own. E2 stimulates a variety of signal transduction molecules that localize to the membrane (2–5, 7, 10, 14), potentially mediated through membrane ER. Signaling could also result from not well defined interactions of E2 or E2/ER with other membrane-localized proteins, such as growth factor receptor tyrosine kinases (epidermal growth factor receptor or IGF-1 receptor) (30–32), or through complex interactions with SHBG (33). A related and developing story is the contributions of signaling by E2/ER to cell biological actions (8–10). However, it is unclear how ER signals and where the endogenous receptor resides within the plasma membrane. The latter is critical information because the spatio-temporal arrangement of signaling molecules is important to the modulation of these cascades.

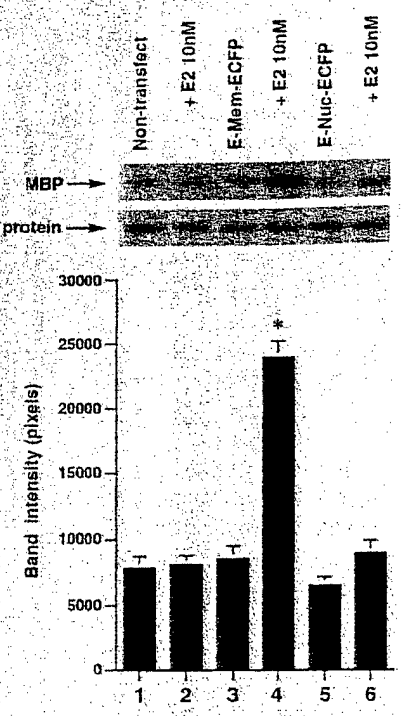


Fig. 7. Transient Expression and Targeting of the E Domain of ERα to the Plasma Membrane of CHO Cells Results in E2 Activation of ERK

CHO cells were not transfected or were transiently transfected to express either E-Mem-ECFP or E-Nuc-ECFP, as described in *Materials and Methods*. ERK activity was then determined in response to an 8-min incubation with 10 nM E2. ERK protein is shown *below* the activity studies, and the *bar graph* data represent three combined experiments. *, $P < 0.05$ for nontransfected CHO (+E2) or E-Mem-ECFP CHO (−E2), *vs.* E-Mem-ECFP CHO (+E2).

Here we report that ER is found in the plasma membrane. Immunocolocalization shown by confocal microscopy indicated extensive, but not complete, overlap of ER with caveolin-1 in the whole-cell plasma membrane. Regarding our immunoblot studies, we determined that ER exists in noncaveolar compartments of the membrane, but predominantly is associated with isolated caveolae vesicles. The latter finding is consistent with a recent report that ER can be localized to caveolar subfractions of the endothelial cell (EC) plasma membrane (34). We additionally illustrate novel findings that ER associates with caveolin proteins in the plasma membrane and that E2 modulates this association differentially, dependent upon cell context. Furthermore, the differential modulation by E2 occurs through signal transduction to ERK via ER. In the MCF-7 cells, association of ER and cav-1 is down-regulated due to ERK activation, whereas in the VSMC, E2 inhibits basal or growth factor-induced ERK and up-regulates ER/cav-1 association. We also show that E2 differentially modulates the production of caveolin, and that this can occur in part through effects on transcription. The latter finding implicates an action of the nuclear receptor. E2 also modulates the stability of caveolin-1, occurring in cell-specific fashion. Finally, we demonstrate that caveolin-1 expression down-regulates E2-induced signal transduction and facilitates ER translocation to the membrane. Both of these functions require the caveolin scaffolding domain for full efficiency.

Caveolin appears to organize the association of signaling molecules within the caveolae (17), including Ras, Src, and PI3K. These molecules move to the membrane from cytoplasm to be activated (35) and are found in caveolae, where they complex and attach to caveolin proteins. Growth factor receptors also localize to the caveolae, where downstream signaling, *e.g.* to ERK, may be facilitated (17). Caveolae dynamics have been elucidated for the activation of endothelial nitric oxide synthase (eNOS). Caveolin-1 attaches to eNOS, keeping the enzyme in a relatively inactive state in the caveolae (36). Activators of eNOS trigger a calcium- and calmodulin-dependent displacement of caveolin-1 from eNOS, leading to the increased activity of the enzyme (37). Also as a result, caveolin translocates out of the plasma membrane. Recently, Chambliss *et al.* (38) reported that ERα and eNOS exist together in EC membrane caveolar fractions, and this facilitates eNOS activation by E2. The results of Chambliss *et al.* seem to be at odds with the aforementioned studies (36, 37), which show that caveolin protein inhibits E2 activation of signaling. However, we believe that the caveolin proteins have a dual purpose in this setting. Serving as a scaffold protein, caveolin helps assemble and localize the signaling molecules into a complex that is capable of being activated. Nevertheless, caveolin itself can inhibit signal activation and may need to dissociate from the assembled complex, as for instance, to allow eNOS activation (36, 37). There is a precedent for this idea with other scaffold proteins. The kinase suppressor of Ras (ksr) purportedly acts as a scaffold protein, forming a complex with MEK, 14–3-3 proteins, ERK, and heat shock proteins 70 and 90, but itself inhibits Ras signaling (39). Similarly, the Jip family of proteins assemble signal molecules in the c-Jun N-terminal kinase (JNK) pathway, thereby modulating JNK activation in the cytoplasm, yet they restrain JNK from translocating to the nucleus and thus inhibit the function of this kinase (40, 41).

E2 has been previously shown to rapidly stimulate NO production in the caveolae (23) through the activation of ERK (10), although it is not clear how ERK participates. We show that blocking E2 activation of ERK prevents the dissociation of ER and caveolin-1 within the MCF-7 membranes. Thus, we propose that the ability of E2 to activate eNOS (10) may result from an ERK-dependent dissociation of ER and caveolin, leading to the activation of NO synthase, perhaps through the recently described activation of PI3K and AKT (42). We also note that, because E2 can regulate caveolin transcription/production, this could influence the longer term interaction of membrane ER and caveolin, promoting localization of ER at the membrane and modulating signal transduction. This likely

represents an example of the coordinated cellular actions of the nuclear and membrane pools of ER, a general concept that has a precedent in other cell models (9, 43).

In our studies, transfection of caveolin-1 cDNA in MCF-7 cells prevented E2 activation of ERK. Overexpression of caveolin-1 inhibits ERK activation by epidermal growth factor (44) and breast cancer cell migration and anchorage-independent growth (45). Transformation of cells by oncogenes is associated with a reduction or loss of caveolin-1 expression (45). Furthermore, stable expression of caveolin-1 antisense in NIH 3T3 cells leads to transformation that is reversed by restoring caveolin-1 protein to normal levels. In this model, hyperactivation of p42/p44 isoforms of ERK resulted from caveolin down-regulation (46). In human breast cancer specimens, endogenous ERK activity is consistently hyperexpressed (47). ERK activation in response to E2/ER action at the membrane significantly contributes to breast cancer cell growth and survival *in vitro* (7, 9). Therefore, the ability of E2 to down-regulate caveolin synthesis and association with ER in MCF-7, leading to the activation of signaling molecules such as ERK, is likely to be important in this regard.

In these same cells, we found that expression of exogenous caveolin-1 caused the loss of ER in the cytosol and an increased amount of ER expressed in the membrane. Although the effects were moderate, this probably reflects the presence of endogenous caveolin protein in the MCF-7 that may have limited the functional effects of overexpression on ER translocation to the membrane. More importantly, the total membrane pool of endogenous ER is only approximately 3% (6, 12), and therefore a limited number of ERs are available to move to the membrane under most circumstances. In a non-overexpression model for ER and caveolin-1, we found binding of E2 at the cell surface and that expressing caveolin-1 into cells that normally do not produce this protein greatly increased this binding, but only at the membrane. Importantly, the lack of enhanced E2 binding to ER in the nuclear fraction of Caco-2 cells rules out an alternative effect of caveolin to enhance E2 binding, through a mechanism apart from facilitating receptor translocation. In all, these data support our proposal that caveolin-1 facilitates the translocation of ER to the membrane, after binding to this receptor in the cytoplasm. In MCF-7, our results also extend the recent findings of Schlegel *et al.* (48), who demonstrated that overexpression of caveolin-1 results in translocation of ER from the cytoplasm to the nucleus. In a similar model in prostate cells, Lu *et al.* (49) have recently shown that caveolin-1 interacts with the AR and facilitates androgen transcriptional activity. We also found translocation of ER from the cytosol to the nucleus after overexpression in MCF-7 (our unpublished results). However, at steady state, 90% of caveolin-1 is at the plasma membrane (23) and, therefore, endogenous caveolin may be most important to facilitate ER movement to this location. Supporting this idea, caveolin-1 enhances the transport of the caveolin-2 protein to the plasma membrane (50).

Recently, Schlegel and Lisanti (51) determined that the plasma membrane attachment and caveolae targeting regions of caveolin-1 reside within the scaffolding domain (residues 82–101) and the first 16 nucleotides of the C-terminal region (135–150). In fact, caveolin-1 that lacks the scaffolding domain is incapable of reaching the cell surface (27). We found that the scaffolding domain is an important contributor to the ability of exogenous caveolin-1 to promote ER translocation, which is consistent with the aforementioned studies. Furthermore, these two proteins associate in the cytoplasm, but this is not dependent upon the presence of this domain. Therefore, we propose that the scaffolding domain facilitates ER localization at the membrane and, therefore, the full-length caveolin protein (but not the scaffolding domain-deleted protein) inhibits ERK activation. Importantly, these data further support the idea that it is the membrane-localized ER that modulates signal transduction cascades to MAPK (see below). The scaffolding domain may also serve a second role to restrain signaling, in some way, at the membrane (52, 53). A detailed analysis of other caveolin, as well as ER, domains that contribute to this process, and the mechanisms facilitating ER translocation and modulation of signal transduction in caveolae, is underway.

A still controversial issue is whether the membrane ER is responsible for the activation of signaling (for instance to ERK) after E2 ligation of endogenous steroid receptors. Previous results suggest that the membrane receptor is important. This is based upon 1) the rapid effects of E2 to stimulate a variety of signaling pathways, 2) the activation of these pathways by a membrane-impermeable E2-BSA compound, and 3) the linkage of the membrane ER to G protein activation and subsequent signaling, an event that is known to occur only at the plasma membrane. Here, we have taken a different approach and found that targeting the expression of the E domain of ERα to the plasma membrane allowed the activation of ERK but did not result in the transactivation of an estrogen response element/luciferase reporter by E2. For comparison, targeting of the E domain to the nucleus did not result in E2-induced MAPK activation. These results suggest that the E domain of the native membrane receptor is important to activate the signaling molecules at the plasma membrane that result in the subsequent activation of ERK (29).

One important finding is that E2 can differentially modulate the production of caveolins and association with ER in the membrane, depending upon cell type. It is well appreciated that a variety of growth-modulating cytokines, including E2, can stimulate proliferation in one cell while inhibiting this process in another. Although this is perhaps related to modulating different signaling cascades in cell autonomous fashion (54–56), the exact details of these responses to proteins

such as TGFβ, platelet-derived growth factor, or angiotensin II is unknown. We propose that, at least for E2, this occurs in part via the differential modulation of caveolin production and association/function. It has been demonstrated that endothelial cell proliferation factors, such as vascular endothelial growth factor and basic fibroblast growth factor, inhibit caveolin-1 synthesis in EC (57), consistent with the effects of E2 reported here. Our findings allow us to hypothesize that the positive or negative modulation of caveolin production and association with signaling molecule complexes may contribute to the differential actions of other growth-regulatory cytokines as well.

As mentioned, the down-regulation of caveolin, and the subsequent activation of signaling, could contribute significantly to E2-induced growth and survival of breast cancer (7, 9). Furthermore, E2-related inhibition of VSMC proliferation and migration has been demonstrated *in vivo* after acute vascular injury (58). This may result from the up-regulation of caveolin dynamics at the membrane, as shown here. Increased caveolin would inhibit growth factor signaling to ERK, providing a mechanism by which E2 inhibits VSMC proliferation (12), and the reactive hyperplasia that results in the injured vessel wall *in vivo* (58, 59).

MATERIALS AND METHODS

Materials

Antibodies and substrate for kinase activity were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). PD 98059 was a gift from Dr. Alan Saltiel (Parke-Davis, Morris Plains, NJ). Caveolin reporter expression reagents were kindly provided by Dr. M. Lisanti (Albert Einstein College of Medicine, New York, NY). Primary cultures of human VSMC were prepared and used as previously described (12). MCF-7 cells were obtained from ATCC (Manassas, VA).

Subcellular Fractionation of Rat Lung Homogenates to Isolate Endothelial Cell Plasma Membranes and Caveolae

The luminal EC plasma membranes and caveolae were isolated directly from rat lung tissue using an *in situ* silica-coating procedure (15, 24). Briefly, the rat lungs were perfused via the pulmonary artery with a colloidal silica solution to coat the surface endothelium. This allowed selective isolation of the EC plasma membranes from the lung homogenate by centrifugation. The caveolae were separated from the membrane by shearing and then isolated by sucrose density centrifugation in a low buoyant density fraction, well separated from the membrane pellet stripped of caveolae.

For translocation and other membrane studies, cells were washed three times with PBS, then lysed in buffer A (50 mM Tris-HCl, pH 7.5; 5 mM EDTA; 100 nM NaCl; 50 mM NaF; 100 μM phenylmethylsulfonyl fluoride; protease inhibitor cocktail; and 0.2% Triton X-100) and sonicated, after which nuclear pellets were collected through low-speed centrifugation. The supernatants were centrifuged at 100,000 × *g* for 30 min to pellet cell membranes. The cell membranes were then further separated by sucrose gradient overlay, and [in accordance with our extensive past experience (15, 16, 24)], fractions 3–5 contained the buoyant membranes (with caveolae and rafts) that were pooled for experiments. Briefly, membrane samples were placed into a tube with an equal volume of 85% (wt/vol) sucrose/25 mM A-morpholine-ethanesulfonic acid and 0.15 M NaCl solution, then overlaid with 8.5 ml of 35% sucrose, topped up with 16% sucrose, and centrifuged at 35,000 rpm for 18 h at 4 C. Ten fractions (1 ml each) were obtained and further processed, or separated on SDS-PAGE followed by membrane transfer for immunoblotting. The membrane fractions were immunoblotted with antibodies to caveolin-1 and 5′NT (plasma membrane proteins), transportin and NTF-2 (nuclear proteins), and β-COP (endosomal/Golgi marker protein). Only caveolin and 5′NT were enriched and found in the early pooled fractions.

Kinase Activity Assays

For ERK activity assays, the cells were synchronized for 24 h in serum, phenol red, and growth factor-free medium. The cells were then exposed to E2, 10 nM, for 8 min with or without additional substances, as previously described (12). Immunoprecipitated kinases were then added to substrate myelin basic protein for *in vitro* assays (6, 9). In some studies, the E domain of ERα was transiently expressed in CHO cells, from plasmid vectors that targeted this portion of the receptor to either the nucleus (E-Nuc-ECFP) or the plasma membrane (E-Mem-ECFP) (29). After recovery, the cells were synchronized without serum for 12 h, after which 10 nM E2 was added to the cells for 8 min, and ERK activity was then determined.

Transient Transfections

VSMC (passage 1–2) or MCF-7 were grown to 50–60% confluence, and then transiently transfected with LipofectAMINE and 1.5 μg of fusion plasmid when cells were cultured in each well of six-well plates (luciferase reporter studies). For all other studies, 10 μg total DNA/100-mm dishes of cells were used. For reporter assays, the plasmids included pA3-Luc, and Pr-750bp, Pr-3kb, and Pr-3 kb and Int 1/pA3 Luc, which contain various lengths of the caveolin-1 promoter, upstream of the ATG site and cloned into a luciferase reporter (pA3-luciferase) (26). In other experiments, 10 μg pCB7Cav-1 (full length), which expresses canine caveolin-1, or pCB7Cav-1 (Δ 60–100), which is missing the scaffolding domain segment, were used, except for ERK studies in which 5, 10, and 15 μg plasmid were used. The cells were synchronized and incubated with E2 as previously described (6, 12). Cell extract supernatants were assayed by the dual-luciferase reporter assay system (Promega Corp., Madison, WI). To correct for transfection efficiency, cells were cotransfected with 0.1 μg of pRL-SV40 expressing the Renilla luciferase (Promega Corp.). In other experiments VSMC, MCF-7, or CHO cells were transfected with plasmids expressing a constituitively active MEK-1 (Upstate Biotechnology, Inc., Lake Placid, NY).

Caveolin Synthesis

Cells were serum deprived for 24 h and then incubated in methionine-free DMEM with dialyzed 10% FBS for 1 h before experimentation. The cells were then incubated in the absence of serum or unlabeled methionine, but with 250 μCi of ^{35}S-methionine in the presence or absence of E2 for 8 h. Caveolin-1 or -2 protein was immunoprecipitated from lysed cells, and the proteins were denatured in SDS and resolved by PAGE, followed by fluorography and autoradiography. In additional pulse-chase studies, the cells were labeled for 1 h with ^{35}S-methionine, and then the medium was replaced with 10-fold excess unlabeled methionine, in the presence or absence of 10 nM E2. At intervals over 8 h, the cells were lysed, and caveolin-1 was immunoprecipitated and resolved by PAGE.

Coimmunoprecipitation and Immunoblot Protein Analysis

Membrane and cytosolic fractions were incubated with protein A-Sepharose for 1 h, after which supernatants were transferred to fresh tubes containing protein A-Sepharose conjugated to caveolin proteins and incubated for 4 h at 4 C. Immune complexes were washed and boiled and then separated by SDS-PAGE. After transfer to nitrocellulose, the proteins were washed with blocking solution and incubated with primary antibody to ERα for 2 h and then with horseradish peroxidase-conjugated second antibody (Santa Cruz Biotechnology, Inc.). Bound IgGs were visualized using enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Arlington Heights, IL) and autoradiography. In other experiments, as described previously (15, 24, 25), rat lung protein subfractions were solubilized and separated by SDS-PAGE and transferred to nitrocellulose filters, followed by immunoblotting. Primary antibody (diluted from 1:500 to 1:5,000 in Blotto, Sigma, St. Louis, MO), was followed by the appropriate horseradish peroxidase-labeled reporter antibodies (diluted 1:1,000). Reactivity was visualized using enhanced chemiluminescence and quantified densitometrically using ImageQuant (Quantum Images, San Diego, CA). Protein concentrations were measured using the micro BCA protein assay kit with BSA as a standard following a protocol previously described (24, 25).

Immunofluorescence Microscopy

Bovine aortic endothelial cells were grown on coverslips and then methanol fixed before dual immunofluorescence confocal microscopy was performed (60). Cells were then stained with monoclonal antibody to caveolin (1:250 dilution of clone Z034; Zymed Laboratories, Inc., South San Francisco, CA) and polyclonal antibody to ERα (1:250 dilution of MC20; Santa Cruz Biotechnology, Inc.). Binding of primary antibody was detected by a reporter IgG conjugated to Texas Red (antimouse IgG) and Bodipy (antirabbit IgG) (Molecular Probes, Inc., Eugene, OR).

Binding Studies

Caco-2 cells (rat intestinal epithelial cells from ATCC) were grown on 100-mm petri dishes in DMEM-F12 without phenol red. Twenty four hours after transfection with 5 μg of pcDNA3-ERα (plus 5 μg of backbone vector), or with 5 μg each of both ERα and caveolin-1 constructs, the cultures were washed, and lysed in buffer A (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 100 nM NaCl, 50 mM NaF, 100 μM phenylmethylsulfonyl fluoride, protease inhibitor cocktail, and 0.2% Triton X-100). Nuclear pellets were collected through low-speed centrifugation. The supernatants were centrifuged at 100,000 × *g* for 30 min to pellet cell membranes. Both pellets were washed twice, once without detergent. Fifty microliters of membrane proteins from the cells were incubated with/without 1 μM unlabeled E2 but always with 50 μl ^{3}H-E2 (specific activity, 80 Ci/mmol, pH 7.5) (Perkin-Elmer Corp., Norwalk, CT) (1.4 pmol of labeled steroid) at 37 C for 45 min, as previously described by us (6). The pellets were washed three times and then quantified by β-scintillation counting. The differences in the presence and absence of unlabeled E2 constituted specific binding.

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Rapid actions of plasma membrane estrogen receptors

Martin J. Kelly and Ellis R. Levin

Functional evidence for the existence of plasma membrane estrogen receptors in a variety of cell types continues to accumulate. Many of these functions originate from rapid signaling events, transduced in response to 17β-estradiol (E_2). It has been convincingly shown that E_2 activates phosphoinositol 3-kinase and protein kinase B/AKT, and stimulates ERK and p38 MAP kinases. In part, this stems from G-protein activation and the resulting calcium flux. As a result, the link between E_2 action at the cell membrane and discrete biological actions in the cell has been strengthened. There is now convincing *in vitro* evidence that E_2 can modulate the functions of neural and vascular cells via non-genomic actions. Thus, the actions of discrete pools of E_2 receptors are likely to contribute to the overall effects of the sex steroids.

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The concept that steroids can act rapidly at the plasma membrane has been defined most extensively by work with the neuroactive steroids[1], which now include estrogen. The actions of 17β-estradiol (E_2) at the membrane result mainly from the binding of this sex steroid to estrogen receptors (ERs), which rapidly activate cellular signaling systems upon ligation. This generates second messengers and has potential biological consequences in a variety of target cells[2–5]. Signal transduction can occur as the result of E_2 activating G proteins, directly or indirectly (reviewed in Ref. 6). In a transfected Chinese hamster ovary cell model at least, ER can activate $G\alpha_s$ and $G\alpha_q$ in the membrane[7]. G-protein activation leads to the modulation of downstream pathways that have discrete cellular actions, including membrane K^+ channel activation[8]. It is also probable that the plasma membrane ER uses tyrosine kinase signaling pathways for important functions such as cell proliferation[9]. Thus, a variety of interactive pathways can potentially be triggered by E_2 action at the membrane, similar to the effects of the more traditional membrane growth factor receptors.

E_2 modulates Ca^{2+} flux and generates cyclic nucleotides via membrane receptors

In a variety of cell types, numerous investigations continue to support the ability of a membrane ER to regulate Ca^{2+} flux. This regulation occurs (after seconds to a few minutes) in response to E_2 exposure. E_2 can rapidly stimulate the entry of Ca^{2+} into isolated duodenal enterocytes through a phospholipase C (PLC)-dependent mechanism involving store-operated Ca^{2+} channels[10]. The effects are specific, in that they are not seen with progesterone (P_4) or testosterone (T). These results are consistent with previous data demonstrating that E_2 activates PLC at the membrane[11] and stimulates a protein kinase C (PKC) pathway.

The stimulation of intracellular Ca^{2+} ($[Ca^{2+}]_i$) flux via membrane ER is also proposed to underlie the important ability of the sex steroid to stimulate nitric oxide (NO) formation in monocytes. In these cells, 1 nM E_2 stimulates a transient increase in $[Ca^{2+}]_i$, followed by NO release, with both events occurring within 40 sec (Ref. 12). These effects are blocked by tamoxifen but not by ICI 182,780. In female colon, E_2 (but not P_4 nor T) increases $[Ca^{2+}]_i$, and this is dependent upon the activation of L-type, voltage-gated Ca^{2+} channel opening[13]. This leads to the rapid activation of cAMP-dependent protein kinase (PKA), which is found in membrane and cytosolic fractions. In addition, E_2 directly activates PKC in these cells, which contributes to PKA activation. Previously, E_2 has been shown to activate PKC and PKA cascades in hypothalamic neurons, altering synaptic transmission in these cells[14].

E_2 can also stimulate the production of cyclic nucleotides in pancreatic β cells, which produce insulin[15]. E_2 augments glucose-induced increases in intracellular Ca^{2+} and cGMP levels. These effects occur within several minutes of the addition of E_2 to the incubation medium, and are not blocked by inhibiting the soluble (cytosolic) guanylate cyclase (GC). This suggests that coupling of ER to particulate (membrane) GC occurs in these cells.

E_2 or the cell-impermeant compound, E_2–BSA (bovine serum albumin), also rapidly stimulates cGMP formation and NO production, and activates extracellular-regulated kinase (ERK) in human endothelial cells (ECs)[16]. Cell-surface binding of labeled E_2–BSA, presumably to a membrane ER, is also demonstrated in this study. Recently, E_2 has been shown to activate a signal transduction pathway involving p38 mitogen-activated protein (MAP) kinase, leading to the activation of MAP kinase-protein kinase 2 (MAPKAP-2) and the subsequent rapid phosphorylation of heat shock protein 27. These events are essential to the ability of E_2 to preserve EC–cytoskeletal integrity and survival after metabolic and hypoxic insult, and to promote EC migration and primitive capillary formation[17].

The ability of E_2 to stimulate cyclic nucleotides and NO is also evident in blood vessels. Nanomolar amounts of E_2 potentiate vasorelaxation of porcine coronary arteries, in both a cycloheximide- (a protein

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synthesis inhibitor) and an actinomycin- (a DNA synthesis inhibitor) independent fashion[18]. Vasorelaxation was related to the ability of E_2 to stimulate rapid cAMP generation. Using uterine artery myocytes as a model, nanomolar amounts of E_2 were shown to stimulate large conductance, Ca^{2+}-activated K^+ (maxi-K^+) channel opening rapidly and potently, via NO and cGMP (Ref. 19). Recently, Valverde *et al.*[20] have shown that the maxi-K^+ channel, specifically the regulatory β-subunit, is a direct target of the actions of E_2. When the α- and β-subunits of this channel were coexpressed in *Xenopus laevis* oocytes, there was a pronounced E_2-induced increase in maxi-K^+ channel current (~50-fold increase in the probability of opening) in inside-out macropatches[20]. An important qualifier is that these effects were seen only at micromolar (i.e. pharmacological) concentrations of E_2, and, therefore, the relevance for steroid physiology is unclear.

In human sperm membranes, rapid activation of intracellular Ca^{2+} and protein tyrosine phosphorylation occurs in response to E_2 (Ref. 21). E_2 signaling can result in the inhibition of the acrosome reaction induced by progesterone[21]. This is blocked by an antibody to the ligand-binding domain of ER (H222)[22]. The authors of this study also reported the presence of a 29-kDa ER in the membrane, which was phosphorylated in response to E_2. In another study, E_2 or E_2–BSA inhibited gonadotropin-induced androgen secretion from fish testicular fragments, in less than 5 min (Ref. 23). These authors found a high-affinity, saturable membrane ER, the androgen-modulating function of which was inhibited by antiestrogens[23].

Effects of E_2 on membrane channels in the central nervous system

The effects of E_2 on the hypothalamus and anterior pituitary act in concert with its effects on other tissues (e.g. ovary, uterus) to ensure a single ovulatory event that is precisely timed. In the 1970s, it was found that hypothalamic neurons were rapidly (within seconds) inhibited by inotophoretically applied E_2 (Refs 24–26). Subsequently, it was shown that E_2-mediated hyperpolarization of hypothalamic gonadotropin-releasing hormone (GnRH) and amygdala neurons is caused by the opening of an inwardly rectifying K^+ channel[27–29]. Recently, it has been found that E_2 has a similar effect on preoptic neurons, so that the coupling of the E_2 receptor to K^+ channels might be ubiquitous throughout the central nervous system (CNS)[30]. The rapid effects of E_2 on GnRH neurons might be responsible, in part, for the negative feedback control of E_2 on the hypothalamic–pituitary–gonadal axis[28,29].

Outside of the hypothalamus, probably one of the most important target areas for E_2 is the hippocampus, a structure that is involved in learning and memory. Postmenopausal women on hormone replacement therapy show a significantly lower incidence of Alzheimer's disease than women who do not maintain their hormone levels[31]. Recently, Foy and colleagues[32] have shown that, at the cellular level, E_2 enhances *N*-methyl-D-aspartate (NMDA)-mediated long-term potentiation in hippocampal slices. Interestingly, E_2 modulates non-NMDA (kainate)-mediated excitation of hippocampal neurons via activation of the cAMP–PKA pathway[33,34]. Thus, E_2 activates the same cellular pathways (i.e. cAMP–PKA) to augment the excitatory glutamatergic transmission in the hippocampus as have been implicated in the augmentation of inhibitory opioid transmission in the hypothalamus[35].

Effects of E_2 on membrane channels in peripheral excitable cells

Pharmacological concentrations of E_2 can rapidly inhibit L-type Ca^{2+} currents and shorten the action potential duration in guinea pig ventricular and atrial myocytes[36,37]. Micromolar concentrations of E_2 inhibit 80% of the L-type Ca^{2+} current in isolated myocytes. These acute effects of E_2 are reversible and are mimicked by diethylstilbesterol (a synthetic estrogen) and ethinyl estradiol, but not by testosterone or progesterone[37]. Nakajima and colleagues[37], using GTPβS in whole-cell patch pipettes to inhibit G-protein coupling, were unable to block the effects of E_2, so the inhibition of this L-type Ca^{2+} current does not appear to involve a G-protein-coupled action. In addition, in cardiac myocytes, the actions of E_2 do not involve NO because N^G-monoethyl-L-arginine, an NO synthesis inhibitor, does not block the actions of E_2 (Ref. 37). This is in contrast to pulmonary artery endothelial cells, in which E_2 increases NO synthase activity to cause vasodilation[38], and to the findings of Mermelstein *et al.*[39] in the striatum, where the inhibition of L-type Ca^{2+} currents in medium spiny GABAergic neurons by E_2 is via a pertussis toxin-sensitive G protein.

In coronary vascular smooth muscle cells, E_2 and environmental estrogenic pollutants such as DDT inhibit L-type Ca^{2+} channels and activate an outward K^+ current, which results in vasodilation[40]. Hence, E_2 signals through different mechanisms depending on cell context. These studies further suggest the potential importance of the rapid antiarrhythmic (Ca^{2+} channel inhibition) and vasodilatory actions of E_2, although again some of these effects are pharmacological.

Some data indicate that short-term E_2 replacement improves insulin resistance in postmenopausal women with type 2 diabetes mellitus[41]. The insulinotropic effects of E_2 can be attributed to its augmentation of $[Ca^{2+}]_i$ in pancreatic β cells[42] and its inhibition of K_{ATP} channel activity, the site of action of sulfonylurea drugs[43]. Picomolar (~100 pM) concentrations of E_2 increase bursts of Ca^{2+} spikes, which simultaneously generate $[Ca^{2+}]_i$ oscillations in the presence of glucose. This is not

affected by actinomycin D, an inhibitor of RNA synthesis. In addition, these effects are mimicked by 8-bromoguanosine-cAMP, an activator of PKA, and 8-bromoguanosine-cGMP, an activator of protein kinase G (PKG). Pancreatic β-cell electrophysiological activity is governed by K_{ATP} channels. K_{ATP} channel activity, measured in cell-attached patches, is inhibited by ~90% by 100 pM E_2 within minutes[15,42] and full channel activity is restored within 30 min after discontinuation of steroid application. In addition, a selective competitive inhibitor of PKG significantly attenuates the actions of E_2 on K_{ATP} channel activity[42].

An increment in cGMP levels could be mediated by two possible pathways. One mechanism is the activation of particulate GC after the binding of E_2 to a membrane receptor; such a mechanism has been supported in PC12 cells[43]. The other possible mechanism is via the NO-mediated activation of soluble GC after activation of NO synthase (NOS) by E_2 (Ref. 39). However, in pancreatic β cells, NO also activates K_{ATP} channels and, because E_2 clearly inhibits K_{ATP} channel activity, this second mechanism seems unlikely[15,42]. In endothelial cells, E_2 signals through phosphoinositol 3-kinase and protein kinase B/AKT, leading to the activation of endothelial NOS (eNOS) and prevention of leukocyte accumulation after ischemia-reperfusion injury in mice[44].

Additional mechanisms of signaling by a membrane ER

In a variety of target cells, E_2 serves as both a growth and a survival factor. In breast cancer, E_2 can inhibit the cytotoxicity of chemotherapy, a process that occurs mainly by apoptotic cell death[45]. This appears to be regulated, in part, through synthesis of the antiapoptotic protein, Bcl_2. Ultraviolet irradiation or the chemotherapeutic agent taxol depend upon the activation of c-Jun N-terminal kinase (JNK) to enact apoptosis in several cell types[46,47]. E_2 significantly blocks the activation of JNK by both treatment modalities in breast cancer cells, and this also prevents the inactivating phosphorylation of the antiapoptotic proteins Bcl_2 and Bcl-xl in MCF-7 cells[48]. As a result, procaspase 9 is not cleaved and the activity of the death effector caspases (cysteine proteases) is not enhanced. Independently, E_2 also activates the MAP kinase ERK, which contributes to the cell survival actions of E_2.

Does ER have the capacity to signal through interactions with traditional proteins that reside in the membrane? As a potential G-protein-coupled receptor (GPCR), ER might, in some situations, use growth factor receptor tyrosine kinase proteins to stimulate signal transduction. It is well established that GPCRs, such as the endothelin, angiotensin and thrombin receptors, activate signaling cascades that lead to the activation of Ser–Thr kinases such as ERK (Ref. 49). This occurs, in part, because liganded GPCRs activate the endothelial growth factor receptor (EGFR); there is evidence that E_2 can use this mechanism[50]. For instance, E_2 signals to ERK in breast cancer cells through heparan-bound EGF and the EGFR (Ref. 51). In turn, EGF might require the presence of ER in one or more compartments as a necessary component for signaling to behaviors such as lordosis in female rats[52].

Recent data in cells expressing the gene encoding ER indicate that the insulin-like growth factor type I (IGF-I) receptor (IGF-1R) might contribute to E_2 signaling[53]. The authors demonstrated, mainly in cells co-transfected to express both the genes encoding IGF-1R and ER, that E_2 causes the rapid phosphorylation of IGF-1R, association of the two receptors and subsequent ERK activation. This might be relevant in breast cancer, in which E_2 potentiates the IGF-I-induced production of G1/S cell-cycle components and cell proliferation[54]. Interestingly, activation of an estrogen response element (ERE)–luciferase reporter occurred in response to IGF-I, and required the presence of ER (Ref. 53). This is consistent with the ability of a variety of growth factor receptor tyrosine kinases [such as those for EGF, platelet derived growth factor (PDGF) and IGF] to stimulate the phosphorylation of nuclear ER at Ser118, leading to E_2-independent activation of this receptor[55].

Membrane localization of the plasma membrane ER

In GH 3/B6 pituitary tumor cells, E_2 rapidly induces the intracellular release of Ca^{2+} and the influx of Ca^{2+} through voltage-gated Ca^{2+} channels before prolactin secretion[56,57]. Based on confocal laser microscopy of the binding of E_2–BSA, studies using selective antibodies to ERα (e.g. H222) and the binding of ERα antagonists, the membrane receptor in these pituitary cells appears to be ERα or an isoform of this receptor.

However, in the CNS, on the basis of electrophysiological experiments in specific cell types that do not express ERα and ERβ (e.g. hypothalamic, striatal and hippocampal neurons), and/or in transgenic mice that have these receptors deleted (i.e. ERKO mice), there are many rapid membrane effects of E_2 that cannot be attributed to the binding of the steroid ligand to ERα or ERβ (Refs 29,58). For example, GnRH neurons are hyperpolarized by E_2 with kinetics similar to the actions of classic neurotransmitters[28,29]. However, although these express low abundance ERα and ERβ transcripts[59], they have not been shown to synthesize ER protein[60]. In addition, there is evidence in the CNS for the rapid activation of PKC and PKA pathways in the hypothalamus and hippocampus by E_2 (Refs 61,62), independent of binding to ERα or ERβ. ERβ activates a MAP kinase cascade in cortical and hippocampal neurons, which is thought to be linked to the neuroprotective effects of E_2 in the brain[63–66]. Within minutes, E_2 treatment results in activation of the MAP kinases, and this is not inhibited by the classic

ER antagonist ICI 182,780 (Ref. 64). Therefore, the cumulative evidence from the CNS argues for the existence of another E_2 receptor or an isoform of ERα (or ERβ) that is associated with the membrane.

One of the more problematic questions, however, is how ERα interpolates with the membrane and couples to G-protein and intracellular signaling cascades. In one model, ERα (or ERβ) spans the membrane, enabling E_2 to bind to the extracellular domain. However, there are no obvious sequences that would code for hydrophobic, membrane-spanning regions, at least within the classic ERs. One speculation is that a post-translational lipid modification of ERα (or ERβ) could occur in the endoplasmic reticulum, thereby facilitating the movement of the receptor, possibly in association with a transporter protein, into the membrane. Such modifications are integral to the membrane localization of a variety of proteins[67]. However, there are no classic myristoylation or palmitoylation sites within the N-terminus of either nuclear ERα or ERβ. A definitive examination of this possibility awaits the isolation and sequencing of the membrane ER proteins.

A second and perhaps more compelling model is that, at least for a proportion of the time, this receptor exists entirely within the plasma membrane bilayer, and perhaps does not express an extracellular domain. The observations that E_2 can rapidly trigger NOS activity to generate NO (Ref. 39) are relevant to this issue of receptor localization. eNOS is found within detergent-resistant domains of the plasma membrane known as caveolae[68]. In this compartment, the major structural protein, caveolin-1, attaches to eNOS and helps to keep this enzyme inactive[69]. Activators of eNOS, including E_2, must influence events in the caveolae to cause the activation of this enzyme. Growth factor receptor tyrosine kinases such as PDGFR, as well as G proteins and other signaling molecules, have all been localized to this discrete membrane organelle[70,71]. Moreover, it has recently been shown by immunoblotting that caveolar fractions isolated from the plasma membrane of endothelial cells contain ER (Ref. 72). Thus, perhaps the presence of ER within this or similar plasma membrane domains facilitates signal transduction.

Perspective

It appears that E_2 can interact with distinct, compartmentalized pools of receptors, each having unique effects on cellular physiology. In this way, the cell can accomplish either rapid modifications of protein action (via signaling through membrane ERα, ERβ or another ER), or more prolonged regulation of cell protein synthesis and function (via the actions of the nuclear receptors). As a consequence, rapid and more prolonged steroid hormone action could be exquisitely coordinated. However, it is probable that there are also overlapping functions. It is also possible that the large cytosolic pool of ER, envisioned as a reservoir before it moves to the nucleus, can in fact act on cytosolic organelles. In this respect, Zheng and Ramirez showed that E_2 can perturb mitochondrial function[73], perhaps through the actions of a local pool of ER. The challenges in the near future are to continue to identify the discrete actions of the membrane receptors, and to develop membrane-specific agonists and antagonists to delineate and modify discrete cellular functions of this pleiotropic steroid hormone.

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