Bovine Serum Albumin-Estrogen Compounds Differentially Alter Gonadotropin-Releasing Hormone-1 Neuronal Activity

Jennifer L. Temple and Susan Wray

Steroid hormones regulate a host of physiological processes and behaviors. These actions can occur by genomic mechanisms involving gene transcription or by nongenomic mechanisms proposed to involve receptors associated with the plasma membrane. BSA-conjugated steroid hormones have been extensively used to elucidate signal transduction pathways for these hormones. We have previously shown, using calcium imaging, that 17β-estradiol (E2) significantly increases GnRH-1 neuronal activity. During the course of these experiments, it became apparent that three different BSA-estrogen compounds have been used in a variety of cell types: 17β-estradiol 6-O-carboxymethylxime-BSA (E2-6-BSA); 1,3,5(10)-estratrien-3,16α,17β-triol-6-one 6-O-carboxymethylxime-BSA (E-6-BSA); and 1,3,5(10)-estratrien-3,17β-diol 17-hemisuccinate-BSA (E2-17-BSA). The effects of these compounds on GnRH-1 neuronal activity were compared using calcium imaging. E-6-BSA and E2-17-BSA, but not E2-6-BSA, significantly increased all parameters of GnRH-1 neuronal activity. In addition, the effects of these two BSA compounds were reversed by the estrogen receptor antagonist ICI 182,780 but not by inhibition of gene transcription. The effects of E2-17-BSA, but not E-6-BSA were reversed by treatment with pertussis toxin, which blocks G protein-coupled receptors. These data indicate that these compounds cannot be used interchangeably and clearly have different binding properties and/or different effects on target tissues. (Endocrinology 146: 558–563, 2005)

ESTROGEN HAS BEEN traditionally described as a transcription factor, acting within the nucleus of target cells to regulate transcription of estrogen-sensitive genes (1). In addition, estrogen is known to have very rapid effects that are likely to be mediated by nongenomic mechanisms (2, 3). These nongenomic effects potentially occur through classical estrogen receptors (ERα and ERβ) targeted to the plasma membrane (4) or through recently described membrane-associated ERs (5).

To distinguish membrane and intracellular effects of estrogen, many studies have used BSA-conjugated estrogen compounds. These compounds consist of a BSA molecule surrounded by a number of estrogen molecules (32–38 mol of estrogen) conjugated to the BSA at a specific site. Importantly, there are multiple BSA-estrogen compounds available, and used, that differ in the type of estrogen and the site of BSA conjugation. These compounds are used because the BSA renders the estrogen membrane impermeable, and thus, effects observed are attributed to actions through membrane receptors. However, the exact BSA-estrogen used in a study is often not specified, and/or the reason one BSA-estrogen compound is chosen over another is not clarified. Many studies, including a previous study from our lab (6), use BSA-estradiol conjugated at the sixth carbon. However, studies using BSA-estradiol conjugated at the 17th carbon (3, 7) or third carbon (8) and BSA-estril conjugated at the sixth carbon (9) have been published, supporting membrane effects of estrogen.

A report by Stevis et al. in 1999 (10) described potential problems with using these conjugates, including activity not observed with estrogen itself and lack of binding to ERα and ERβ. However, novel ERs have since been identified, and the use of these conjugates has persisted to isolate membrane actions of estrogen (11). Multiple BSA-estrogen compounds are available and often used without rationale of choice, and often, free estrogen levels are not monitored. To investigate the potential differences among the BSA-estrogen compounds, the effects of three such compounds on GnRH-1 neuronal activity were examined. Recently we reported that estradiol (E2) significantly increases GnRH-1 neuronal activity (6). In this study, we show that the effects of the compounds vary greatly depending on the location of the BSA conjugation and the type of estrogen that is conjugated.

Materials and Methods

Materials

For preparation of nasal explants, BSA, d-glucose, apo-transferrin, putrescine, sodium selenite, bovine insulin, l-ascorbic acid, fluoride-oxyuridine, and thrombin were purchased from Sigma Chemical Co. (St. Louis, MO). Gey’s balanced salt solution, Ham’s F-12 nutrient mixture, t-glutamine, and penicillin-streptomycin-nystatin antibiotic mixture were supplied by Invitrogen (Grand Island, NY). Chicken plasma was purchased from Cocalico Biologicals, Inc. (Reamstown, PA). For treatment of nasal explants, 17β-estradiol 6-O-carboxymethylxime-BSA

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Abbreviations: DRB, 5,6-Dichlorobenzimidazole 1-β-D-ribofurano-side; E2, estradiol; E-6-BSA, 1,3,5(10)-estratrien-3,16α,17β-triol-6-one 6-O-carboxymethylxime-BSA; E2-6-BSA, 17β-estradiol, 17β-triol-6-O-6-carboxymethylxime-BSA; E2-17-BSA, 1,3,5(10)-estratrien-3,17β-diol 17-hemisuccinate-BSA; ER, estrogen receptor; PTX, pertussis toxin; SFM, serum-free medium; TTX, tetrodotoxin.

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Nasal explant preparation

Nasal explants were prepared as described previously (12). Briefly, timed-pregnant NIH Swiss mice were obtained and embryos removed at embryonic d 11.5 in accordance with National Institutes of Health guidelines. Nasal regions were dissected under aseptic conditions and refrigerated in glucose-enriched Gey’s balanced salt solution. Explants were adhered to cover slips via a plasma-thrombin clot and maintained in a defined serum-free medium (SFM) at 37°C in a humidified incubator with 5% CO2. On the third day in vitro, nasal explants were treated with fluoroodeoxyuridine (8 × 10⁻⁵ M) for 3 d to inhibit cell proliferation. On the sixth day in vitro, and every 2–3 d after, explants were provided with fresh SFM. The explants were used for experiments on culture d 6–8 or 20–22.

Calcium imaging

Calcium imaging was conducted using previously described methods (13). Briefly, nasal explants were exposed to the indicator dye Calcium Green-1 AM (2.5 μM in 80% dimethylsulfoxide/20% pluronic dye, 1:120) for 20 min in a humidified chamber. Explants were then washed twice with fresh SFM (10 min each) and transferred to a heated perfusion chamber (37.5°C; Warner Instruments, Hamden, CT). Medium was perfused across the explant at a rate of 100 μl/min via a peristaltic pump (Spectra Hardware, Inc., Westmoreland City, PA). Intracellular calcium oscillations were visualized using a Nikon microscope equipped with a ×20 fluorescence objective and an intensified charge-coupled device camera (Video Scope International, Sterling, VA). The camera shutter was controlled by a G4 Macintosh computer via imaging software (IP Labs, Scanalytics Corp., Vienna, VA). Excitation wavelengths were 450–490 nm and emission was monitored from 520–560 nm.

Analysis of calcium imaging data

Intracellular calcium was monitored every 20 sec for 100 min. At the end of the 100-min period, explants were exposed to an acute dose of KCl (20 mM) to cause an abrupt increase in intracellular calcium to confirm the viability of the cells. Using IP Labs, ODs were measured within each cell and background values were subtracted; thus, the corrected OD values represent only intracellular events. The traces of intracellular calcium were then analyzed by PULSAR (14) to determine when calcium peaks occurred (OD readings 2 sd above baseline). Calcium peaks reflect mobilization of intracellular calcium, and as such, are an index of neuronal activity (15). Thus, three of the parameters reported, number of cells with peaks, number of peaks per cell, and mean peak amplitude, are a reflection of neuronal activity in individual GnRH cells in each group sampled. The fourth parameter reported, cells with more than 10 peaks, provides information about GnRH neuronal activity within the sampled population. Each of these parameters are obtained directly from the PULSAR analyzed data.

To provide additional information about neuronal activity in the GnRH population, peak data are further evaluated for patterned activity. Time points when peaks were detected in each cell were compiled into a single file for the entire neuronal population within the imaged field. The MATLAB program divided these data into 1-min intervals and assigned either a 0 (no calcium peaks detected during that 1-min period) or a 1 (>5 significant calcium peak detected). These data were then transformed by the WAVELET analysis program to determine when calcium pulses occurred. A pulse was defined as a period of time when multiple GnRH-1 neurons displayed synchronous calcium oscillations. For each pulse, the duration from start to finish, the interpulse interval (the time from the start of one pulse to the start of the next pulse), and the number of cells contributing to the pulse were determined.

Immunocytochemistry

At the end of the data collection period, explants were fixed with 4% formaldehyde in PBS for 1 h. They were rinsed with PBS and incubated for 1 h in 10% normal goat serum with 0.1% sodium azide and 0.3% Triton X-100 followed by several rinses in PBS. Explants were then incubated overnight at 4°C in GnRH-1 antibody (SW1, 1:3000) (16). The next day, explants were washed with PBS and incubated in goat anti-rabbit conjugated Cy3 (1:800; Jackson ImmunoResearch, West Grove, PA). The staining was then compared with the calcium dye labeling to determine which cells were GnRH-1 cells. Any cells that were not GnRH-1 positive were removed from further analysis (< 5%).

Transfections of MCF-7 cells

To assure that any effects of the BSA-estrogen compounds are not because of unconjugated estrogen, MCF-7 breast cancer cells were transfected with an estrogen responsive construct to determine transfection efficiency. All BSA-estrogen compounds were linked to a luciferase reporter gene (17). Using the manufacturer’s (Invitrogen) instructions, lipofectin was used to transfect this construct into MCF-7 cells. Briefly, cells were grown to 80–90% confluency in six-well plates. For each well, 4 μg of DNA was brought up to 250 μl in OptiMEM medium. In a separate tube, 10 μl of lipofectin was also brought up to 250 μl in OptiMEM. After 10 min, the DNA and lipofectin mixtures were combined and allowed to incubate (20 min). This mixture was then added to 1.5 ml of OptiMEM (per well), and 2 ml of DNA-lipofectin construct was added to each well. After transfection (6–8 h), the cells were treated with E2, E-6-BSA, E-17-BSA, or no hormone for 18 h. Cells were lysed, and their contents were harvested and assayed for luciferase activity. The only way for the luciferase to become activated is for estrogen to bind to an intracellular ER, which then binds to the estrogen response element. Therefore, the relative amount of luciferase activity accurately reflects free estrogen.

Experimental methods for nasal explants

Experimental groups consisted of two to four explants per treatment with an average of 13.8 ± 1.0 (range, 8–32) cells visualized per explant. The sodium channel blocker TTX was used to isolate GnRH-1 neurons from neuronal input to examine direct effects of BSA-estrogen compounds on intracellular calcium oscillations. All drugs were continuously perfused across the explant for the duration of the experiment and, where indicated, explants were treated after calcium dye loading before imaging. The following treatments were given in the presence of TTX (10⁻⁷ M in acetate buffer): E2-6-BSA (2 × 10⁻⁷ M), E-6-BSA (2 × 10⁻⁷ M), E2-17-BSA (2 × 10⁻⁷ M), E-6-BSA plus PTX (500 μM), and PTX (500 μM). For compound treatment, GnRH neuronal activity and the effect was not reversed by DRB treatment, additional experiments were performed using PTX to inhibit G protein-coupled receptors (19). To remove free estrogen, all BSA-estrogen compounds were dissolved in 50 mM Tris-HCl (pH 8.5) and filtered through a 3-kDa cutoff filter (Amicon, Beverly, MA) immediately before use (10). The retentate was resuspended in SFM to its final concentration (2 × 10⁻⁴ M). All doses were based on previously reported dose-response studies using E2 (6).

Statistical analysis

Statistical comparisons of frequency data were calculated using χ² analysis. Comparisons of pulse duration, pulse amplitude, interpulse interval, peak amplitude, number of calcium peaks per cell, and relative luciferase activity were made using ANOVA, followed by a Fisher’s least significant difference post hoc test. All analyses were conducted using Statview statistical software (Abacus Concepts, Inc., Berkeley, CA).
χ² data were considered significantly different if \( P < 0.01 \). The ANOVA data were considered significantly different if \( P < 0.05 \). All data are expressed as mean ± SEM.

**Results**

**TTX plus E2**

Treatment with TTX alone reduced GnRH-1 neuronal activity to baseline levels (Table 1). As previously reported, TTX plus E2 increased the number of calcium peaks per cell, the mean peak amplitude, the percentage of cells with at least 10 peaks, and the percentage of cells contributing to pulses relative to TTX alone (Table 1). These effects were inhibited by treatment with ICI 182,780 and reversed by pretreatment with DRB (6) (Table 1).

**E2-6-BSA**

E2-6-BSA did not mimic most of the effects of E2. There was, however, a significant increase in the mean peak amplitude and the percentage of cells contributing to pulses (Table 1 and Fig. 1). As previously reported, these effects were attributable to a small amount of free E2 in the preparation and were reversed by cotreatment with ICI 182,780 (6) (Table 1).

**E-6-BSA**

E-6-BSA plus TTX treatment increased the number of calcium peaks per cell, the percentage of cells with at least 10 peaks, the mean peak amplitude, and the percentage of cells that contribute to each calcium pulse, relative to TTX-treated explants (Table 1 and Fig. 1). All of these effects were reversed by treatment with the ER antagonist ICI 182,780 except for the percentage of cells contributing to pulses (Table 1). By contrast, treatment with DRB or PTX had no effect on any E-6-BSA measurements except the mean peak amplitude, which was inhibited by both treatments (Table 1). These effects did not differ with age of the explant (data not shown); therefore, the data from both age groups have been combined.

**MCF-7 cell transfection**

When E2 was applied to the transfected MCF-7 cells, there was a 2519% increase in relative luciferase activity compared with the control cells. By contrast, the BSA-conjugated estrogens all showed a slight increase relative to controls, but the only group that was statistically different from controls was E2-17-BSA, with a 270% increase.

**Explants without TTX**

As previously reported, the E2-6-BSA was not different from SFM on any measure except the mean peak amplitude (6). The other two compounds, however, exhibited increases in all parameters above the level of SFM alone (Table 2). This effect was not seen with E2 alone (6).

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**TABLE 1. Summary of calcium imaging data from nasal explants treated with different BSA-estrogen compounds in the presence of TTX**

<table>
<thead>
<tr>
<th></th>
<th>No. of cells with peaks (%)</th>
<th>No. of peaks per cell</th>
<th>Mean peak amplitude</th>
<th>Cells with ≥10 peaks (%)</th>
<th>% cells contributing to pulses</th>
</tr>
</thead>
<tbody>
<tr>
<td>TTX</td>
<td>60/62 (97)</td>
<td>4.4 ± 0.3</td>
<td>13.5 ± 1.2</td>
<td>2/62 (3)</td>
<td>41.7</td>
</tr>
<tr>
<td>TTX plus E2</td>
<td>58/59 (98)</td>
<td>11.5 ± 0.8</td>
<td>20.5 ± 0.6</td>
<td>34/59 (58)</td>
<td>65.8</td>
</tr>
<tr>
<td>Plus ICI 182,780</td>
<td>38/39 (97)</td>
<td>4.6 ± 0.6</td>
<td>17.4 ± 0.9</td>
<td>4/39 (10)</td>
<td>59.9</td>
</tr>
<tr>
<td>Plus DRB</td>
<td>41/44 (93)</td>
<td>2.9 ± 0.3</td>
<td>14.1 ± 0.7</td>
<td>1/44 (2)</td>
<td>44.2</td>
</tr>
<tr>
<td>TTX plus E2-6-BSA</td>
<td>96/96 (100)</td>
<td>5.1 ± 0.3</td>
<td>20.1 ± 0.7</td>
<td>7/96 (7)</td>
<td>62.8</td>
</tr>
<tr>
<td>Plus ICI 182,780</td>
<td>20/21 (95)</td>
<td>3.7 ± 0.3</td>
<td>16.7 ± 0.8</td>
<td>0/21 (0)</td>
<td>41.2</td>
</tr>
<tr>
<td>TTX plus E-6-BSA</td>
<td>8/39 (21)</td>
<td>13.7 ± 1.0</td>
<td>22.5 ± 0.7</td>
<td>51/82 (62)</td>
<td>75.2</td>
</tr>
<tr>
<td>Plus ICI 182,780</td>
<td>24/10 (10)</td>
<td>4.1 ± 0.5</td>
<td>14.3 ± 0.4</td>
<td>0/24 (0)</td>
<td>62.3</td>
</tr>
<tr>
<td>Plus DRB</td>
<td>54/54 (100)</td>
<td>10.3 ± 0.8</td>
<td>12.8 ± 0.5</td>
<td>22/54 (41)</td>
<td>79.1</td>
</tr>
<tr>
<td>Plus PTX</td>
<td>40/41 (98)</td>
<td>11.3 ± 1.7</td>
<td>17.1 ± 0.5</td>
<td>16/41 (39)</td>
<td>82.3</td>
</tr>
<tr>
<td>TTX plus E2-17-BSA</td>
<td>44/45 (97)</td>
<td>12.4 ± 1.2</td>
<td>18.7 ± 0.5</td>
<td>22/45 (49)</td>
<td>70.8</td>
</tr>
<tr>
<td>Plus ICI 182,780</td>
<td>20/21 (95)</td>
<td>7.9 ± 1.0</td>
<td>19.1 ± 0.5</td>
<td>7/21 (33)</td>
<td>49.2</td>
</tr>
<tr>
<td>Plus DRB</td>
<td>40/41 (98)</td>
<td>26.8 ± 2.7</td>
<td>20.4 ± 0.7</td>
<td>34/41 (83)</td>
<td>76.6</td>
</tr>
<tr>
<td>Plus PTX</td>
<td>39/39 (100)</td>
<td>6.6 ± 0.5</td>
<td>19.9 ± 0.4</td>
<td>8/39 (21)</td>
<td>62.6</td>
</tr>
</tbody>
</table>

\( ^a \) Significantly different from TTX (\( P < 0.05 \) for ANOVA and \( P < 0.01 \) for \( \chi^2 \)).

\( ^b \) Significantly different from same TTX plus estrogen/BSA-estrogen treatment (\( P < 0.05 \) for ANOVA and \( P < 0.01 \) for \( \chi^2 \)).
cell across time, and the calcium pulse occurred. The closer to cells (denoted with an asterisk) represents synchronized calcium oscillations across multiple individual examples of calcium imaging traces of 7 d in vitro explants treated with TTX (n = 8 cells) (A), TTX plus E2 (n = 14 cells) (B), TTX plus E2-6-BSA (n = 11 cells) (C), TTX plus E-6-BSA (n = 14 cells) (D), or TTX plus E2-17-BSA (n = 14 cells) (E). The x-axis is time (min). In the black and white section, each row contains data from an individual cell across time, and the white lines represent the time at which a significant peak in intracellular calcium occurred. The colored trace above represents synchronized calcium oscillations across multiple cells (denoted with an asterisk). The more intense the pulsatile event, the closer to red the pulse appears. *, Times at which a synchronized calcium pulse occurred.

Discussion

This study demonstrates that three BSA-estrogen compounds have very different physiological effects on GnRH-1 neuronal activity. The differences among these compounds are an extra hydroxyl group on the E-6-BSA and BSA conjugation at a different position for E2-17-BSA compared with E2 (21). The position of the BSA conjugation at the 17th carbon when these compounds have been used for antibody generation (20). We showed that although the most commonly used BSA-E2 compound, the E2-6-BSA, had only slight effects on GnRH-1 neuronal activity, the other two compounds, which have also been reported in the literature but are used less often, had dramatic effects on all parameters of GnRH-1 neuronal activity that were measured. Additionally, these compounds appear to act through different downstream pathways, as the DRB and PTX had no effect on E-6-BSA, but the effects of E2-17-BSA were inhibited by PTX and enhanced by DRB. The effects of all compounds tested were inhibited by cotreatment with ICI 182,780, suggesting action through an ER.

E2-6-BSA is the compound most often used to investigate potential membrane actions of E2; however, mixed effects have been reported with this compound. For example, in uterine artery endothelial cells and neostriatal neurons, E2-6-BSA mimicked all effects of E2, including inhibition by ICI 182,780 in the endothelial cells (21, 22). In growth plate chondrocytes, E2-6-BSA mimicked the positive effects of E2 on alkaline phosphatase activity and proteoglycan sulfation, but these effects were not reversed by ICI 182,780 (23). In human amnion-like WISH cells, MCF-7 breast cancer cells, and HCC38 cells, E2-6-BSA mimicked some effects of E2 but either to a lesser extent or only on some measures (24, 25). Within GnRH-1 neurons specifically, there are also mixed effects. In GT1–7 cells, which are an immortalized GnRH-1 neuronal cell line, E2-6-BSA mimicked E2 effects on cAMP production (26). In primary GnRH-1 neurons, however, E2-6-BSA had no effect on cAMP response element-binding protein phosphorylation (27) and no major effects on GnRH-1 neuronal activity (6). Importantly, in the latter study, free E2 was measured and found to be present in the preparation after column purification. Therefore, the two parameters (mean peak amplitude and percentage of cells contributing to pulses) that were elevated compared with TTX alone and inhibited by treatment with ICI 182,780 were attributable to the small amount of free E2 (6). Thus, the fact that E2-6-BSA does not consistently mimic E2 may depend on the cell type and the preparation, and when it does, this may be because of unconjugated E2 in the preparation.

Unlike the E2-6-BSA compound, the effects of E-6-BSA on GnRH-1 neuronal activity significantly increased all parameters measured. In addition, the effects were not inhibited by pretreatment with DRB or by cotreatment with PTX. Although this compound is not widely used, there is a recent report of effects in dorsal root ganglia neurons. Specifically, E-6-BSA was found to mimic E2 by blocking ATP-induced calcium currents in these neurons (10). It is important to note that this compound is a BSA-conjugated estradiol and not E2 and therefore has a different affinity and different ER binding properties than E2 (28). To our knowledge, this is the first report of E-6-BSA on GnRH-1 neurons.

The effects of E2-17-BSA were similar to E-6-BSA. Treatment with this compound led to significant increases in GnRH-1 neuronal activity. However, these effects differed from the E-6-BSA compound in that they were enhanced by treatment with DRB and inhibited by treatment with PTX. The increase in GnRH neuronal activity after DRB treatment is intriguing and suggests decreased transcription of a product that, at an unknown level, inhibits the action of E2-17-BSA. Although this compound is not as widely used as E2-6-BSA, it has been used in a variety of hypothalamic preparations where its effects are reported to mimic those of...
TABLE 2. Summary of calcium imaging data from nasal explants treated with different BSA-estrogen compounds

<table>
<thead>
<tr>
<th>Compound</th>
<th>No. of cells with peaks (%)</th>
<th>No. of peaks per cell</th>
<th>Mean peak amplitude</th>
<th>Cells with ≥10 peaks (%)</th>
<th>% Cells contributing to pulses</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFM</td>
<td>43/44 (97)</td>
<td>9.3 ± 1.4</td>
<td>14.4 ± 0.3</td>
<td>14/44 (32)</td>
<td>43.5</td>
</tr>
<tr>
<td>E2</td>
<td>60/63 (95)</td>
<td>6.2 ± 0.8</td>
<td>15.2 ± 0.4</td>
<td>12/63 (19)</td>
<td>39.3</td>
</tr>
<tr>
<td>E2-6-BSA</td>
<td>42/42 (100)</td>
<td>8.2 ± 0.3</td>
<td>21.9 ± 0.6*</td>
<td>10/42 (24)</td>
<td>52.0</td>
</tr>
<tr>
<td>E-6-BSA</td>
<td>28/28 (100)</td>
<td>28.6 ± 5.1*</td>
<td>23.8 ± 0.6*</td>
<td>18/28 (64)*</td>
<td>65.0*</td>
</tr>
<tr>
<td>E2-17-BSA</td>
<td>13/14 (93)</td>
<td>26.0 ± 3.8*</td>
<td>28.0 ± 1.4*</td>
<td>12/14 (86)*</td>
<td>64.2*</td>
</tr>
</tbody>
</table>

* Significantly different from SFM (P < 0.05 for ANOVA and P < 0.01 for χ²).

E2. Specifically, both E2 and E2-17-BSA attenuated the GABA<sub>A</sub> response to baclofen treatment in guinea pig hypothalamic slices (7).

It is becoming increasingly clear that E2 can have very rapid effects on various cell types that are unlikely to be mediated by gene transcription. A proposed pathway by which these effects are exerted begins with an ER that is distinct from nuclear ERs and is associated with the plasma membrane. In the studies reported here, the effects of E-6-BSA and E2-17-BSA were seen at both 7 d in vitro and 21 d in vitro, even though the percentage of cells expressing ERβ is significantly decreased by 21 d in vitro (29). This suggests that these effects are mediated by an ER other than ERβ. There is physiological and immunocytochemical evidence for these receptors (3, 5, 7). However, it has proved challenging to develop selective ligands for them. One group has used a ligand, diphenylcyclopentadienone (STX), that is devoid of estrogenic activity on nuclear ERs and is a significantly more potent activator of rapid estrogenic responses (7). Other than this compound, most studies have relied on BSA-conjugated E2.

In 1999, Stevis et al. (10) reported several problems with using BSA-conjugated estrogen compounds to mimic the effects of E2 at the membrane. They showed that the two compounds tested had effects that did not mimic E2 and were, in fact, greater than those observed with E2. We have shown similar results in a different system in that none of the BSA-conjugated estrogen compounds that were used here directly mimic E2. This leaves several unanswered questions, including whether E2 preferentially acts within the cell when in its native form and not prevented from crossing the cell membrane. Also, is BSA conjugation changing the binding properties of the estrogen and thereby altering their downstream effects and/or activating another receptor system? Finally, although dramatic effects of BSA-conjugated estrogens have been clearly demonstrated, the physiological relevance of such experiments remains to be determined.

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