Endocrine, *vol. 27, no. 3, 253–258, August 2005* 0969–711X/05/27:253–258/$30.00

Potentiation of Genomic Actions of Estrogen by Membrane Actions in MCF-7 Cells and the Involvement of Protein Kinase C Activation

Nino Devidze, Donald W. Pfaff, and Lee-Ming Kow

Laboratory of Neurobiology and Behavior, The Rockefeller University, 1230 York Ave., New York, NY 10021

It is now well established that estrogens (E) have at least two kinds of actions: genomic and nongenomic. But the relationship between these actions has hardly been explored. In this study we investigated this relationship in MCF-7 cells, a human breast cancer cell line, and explored the possible involvement of protein kinase C (PKC) signaling pathways. For this purpose a two-pulse paradigm was used: cells were treated *(1)* with 17β-estradiol (E), E conjugated with bovine serum albumin (E-BSA or fE'), or other test agents in the first pulse and *(2)* with E in the second pulse following a 4-h interval. An E-BSA+E paradigm was used to show that replacement of E with the membrane-impermeable E-BSA in the first pulse could potentiate genomic actions of E, in the second pulse. To investigate involvement of signaling pathways, two PKC activators, phorbol 12,13-diacetate (PDAc) or phorbol 12-myristate 13-acetate (PMA), and inhibitors (chelerythrine chloride and H7-dihydrochloride) were used to replace E or E-BSA in the first pulse. PDAc was as effective as E or E-BSA in potentiating the genomic action of E in the second pulse, while PMA was almost without an effect. Conversely, the potentiating effects of E-BSA and PDAc were blocked by chelerythrine chloride but, interestingly, not by H7. The exact reason underlying these differences is not known. In summary, in MCF-7 cells a membrane action of E can potentiate a later genomic action and involves PKC signaling.

Key Words: 17β-estradiol; PKC isozymes; PKC activator; PKC inhibitor; genomic action; membrane action.

Introduction

One of the important approaches to understanding molecular mechanisms of transcriptional controls has concerned steroid receptors, including estrogen receptors (ERs), members of the nuclear receptor superfamily *(1)*. ERs have significant effects on the regulation of many physiological actions, such as sexual development and reproductive cycle growth, bone mineralization, cardiovascular functions (in both males and females), and brain masculinization (males) *(2–4)*. Earlier studies showed that the regulatory mechanisms of estradiol could be characterized by its genomic actions *(5,6)*. However, its nongenomic actions, mediated by membrane mechanisms, have also been described *(7–10)* and further supported by findings which showed that membrane ERs bind to estradiol and activate a novel G protein that in turn initiates a signal transduction at the cell surface *(11–13)*. In a study using SK-N-BE2C, human neuroblastoma cells that have very little or no constituent ER and that were transiently transfected with human ERα, nongenomic (fast) and genomic (slow) actions of estradiol can synergize to achieve the transcriptional potentiation *(14)*. The same study showed that protein kinase C (PKC) plays a role in the nongenomic mechanisms by which E-BSA potentiated the ERα-mediated genomic effects *(14)*, demonstrating the involvement of different pathways in estrogens effects. PKC comprises a family of 12 related isozymes *(15)* that are divided into three groups: classical or calcium-dependent (PKCα, βI, βII, and γ); the novel or calcium-independent (PKCδ, θ, η, μ, and ε); and atypical PKCs (PKCξ and λ/τ) *(15,16)*. PKC involvement in many different kinds of signaling *(17–19)*, including E's rapid action at the membrane *(20)*, have been demonstrated.

Is the potentiation of the genomic actions by acute estradiol unique to neuroblastoma cells *(14)*, or does it also occur in other cell types? PKC involvement in these two actions of estradiol needs to be investigated. For this purpose we studied a breast cancer cell line MCF-7, which possesses constitutional ERs *(21)*.

Results

Synergism Between Two Pulses of Estradiol (E)

To determine an optimal paradigm, E at 10^{-12}, 10^{-11}, 10^{-10}, 10^{-9}, or 10^{-8} *M* was administered to cells in the two-pulse paradigm with pulse duration ranging from 15 to 60 min. The lowest effective E concentration and pulse duration were found to be 10^{-11} *M* and 15 min, respectively. At higher concentration and/or longer pulse duration, one pulse

Received April 6, 2005; Revised June 2, 2005; Accepted June 10, 2005.

Author to whom all correspondence and reprint requests should be addressed: Dr. Nino Devidze, Laboratory of Neurobiology and Behavior, The Rockefeller University, Box 275, 1230 York Avenue, New York, NY 10021. E-mail: devidzn@rockefeller.edu

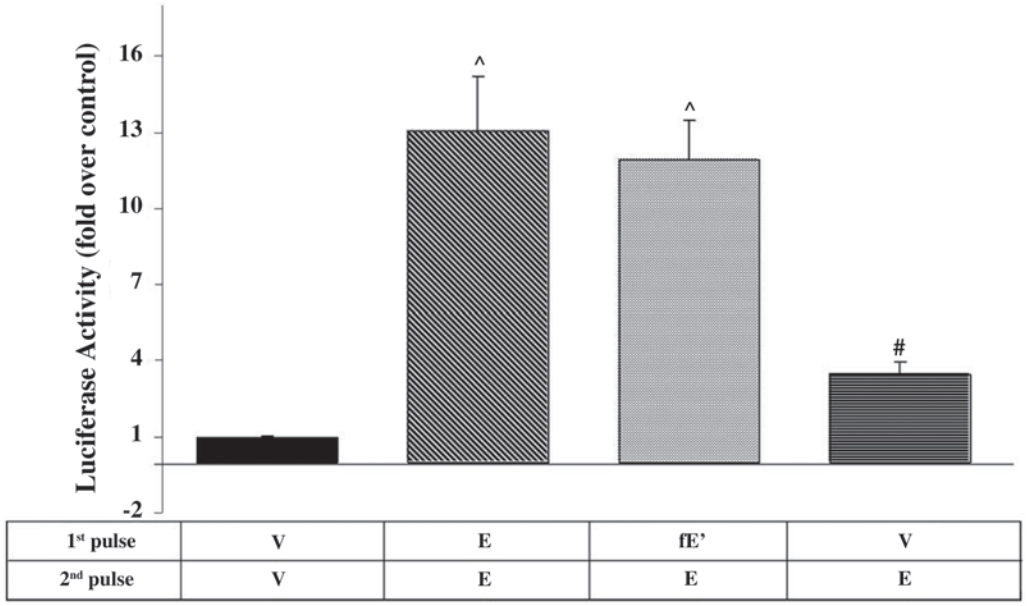


Fig. 1. Estradiol (E) administered in two pulses (E+E) rather than one pulse (V+E) increases transcriptional activity by 12-fold and demonstrated that two pulses of estradiol act synergistically. Replacement of E with filtered E-BSA (fE') in the first pulse (fE'+E) increased transcriptional activity by 11-fold, demonstrating that estrogen's membrane actions can potentiate its genomic actions. Cells were transfected and treated as described in the Materials and Methods section. The means represent samples from two replicate experiments ($n = 4$ per treatment group in each replicate experiment). $p < 0.05$ was regarded as significant. #Indicates significance at $p < 0.005$ and ^indicates significance at $p < 0.0005$ as compared with the control group (V+V).

of E (with vehicle in the other pulse) was often sufficient to induce transcription. Therefore, we chose to use E at 10^{-11} *M*, with a pulse duration of 15 min for the remainder of our study. Compared to the control group, where vehicles were applied in both pulses (V+V group), the group where E was used only in the second pulse (V+E) showed slightly increased transcriptional activity (3.5-fold over the control, $p < 0.005$). In contrast, the use of estradiol in both pulses (E+E) increased transcription rate by 12-fold ($p < 0.0005$) over the control (Fig. 1). Thus, two pulses of E can act synergistically in achieving transcriptional function.

Membrane Impermeable Estradiol Is as Effective as Estradiol in Potentiating Estrogen's Genomic Action

To explore the possibility that membrane actions of estrogen can potentiate its genomic action, the E in the first pulse was replaced with filtered E-BSA (fE') that would limit the estrogen action to the cell membrane. As in the E+E group, the combination of fE' in the first pulse and E in the second pulse (fE'+E) also induced an increase in the transcription of the luciferase reporter gene by 11-fold ($p < 0.0005$) over the control group (Fig. 1). There is no difference statistically in the increases between E+E and fE'+E groups. These results indicate that estrogen's membrane actions can potentiate its genomic actions.

PKC Activation Can Mimic the Transcription-Potentiating Actions of E-BSA

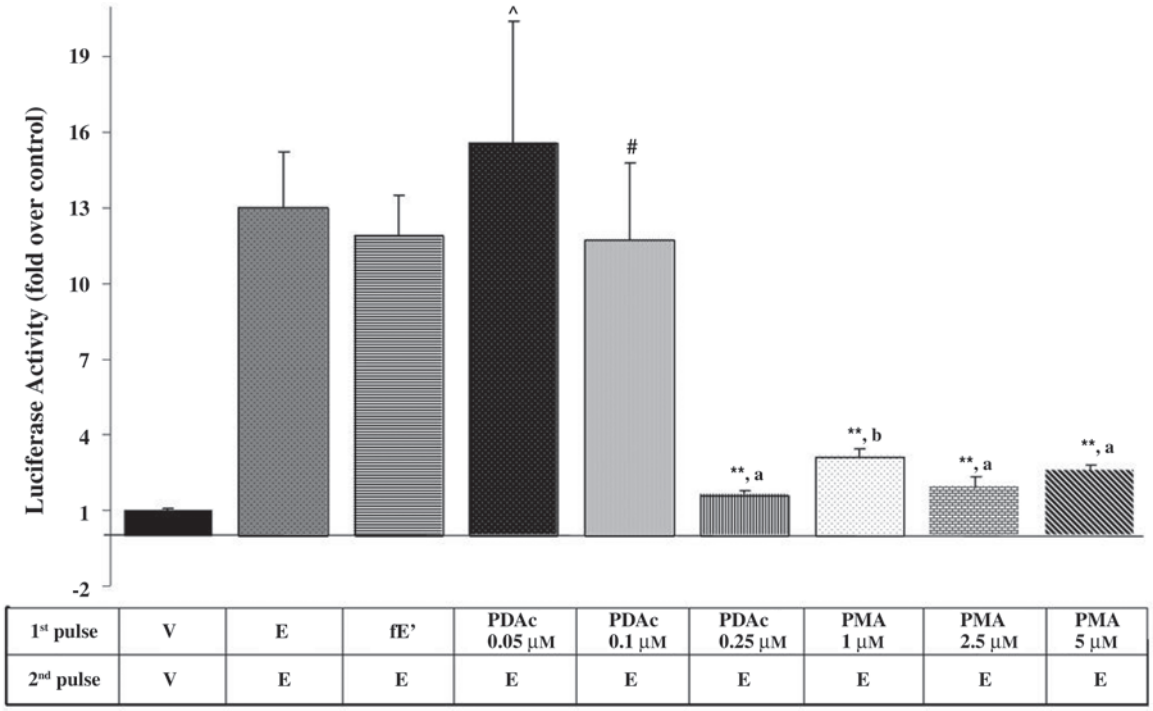
The involvement of transcriptional signaling pathways in actions of estradiol in MCF-7 cells was studied by using PKC inhibitors and activators. To determine whether PKC activation can mimic acute estrogen actions, two PKC activators—PMA and PDAc—were employed in the first pulse and estradiol in the second. Administration of PMA at 1, 2.5, and 5 μ*M* did not potentiate the transcriptional effect of estrogen (Fig. 2). This was not because the PMA used was inactive, as it was able to stimulate the expression of β-gal [the difference in β-gal activity was significantly higher when it was compared to all of the concentrations of PDAc ($p < 0.002$)].

In contrast to PMA, PDAc was as effective as E or fE' in potentiating estrogen's transcriptional action (Fig. 2). At the lowest concentration used (0.05 μ*M*), PDAc combined with E caused a 15-fold increase ($p < 0.0005$) in the transcription of the luciferase reporter gene (Fig. 2).

As the concentration of PDAc was increased to 0.1 μ*M*, the potentiation was reduced to 11-fold that of the control. This reduction was probably seen because PDAc started to become toxic around this concentration, since a concentration of 0.25 μ*M* PDAc caused floating dead cells and a severe decrease in confluence.

PKC Inhibition Can Block Estrogen's Transcription-Potentiating Membrane Actions

To determine whether the activation of PKC is required for the potentiating actions, cells were treated with PKC inhibitors, chelerythrine chloride (CH, 30 μ*M*), or H7 (10 and 50 μ*M*), 30 min before and during the first estrogen treatment pulse, and then washed away. Administration of



1st pulse	V	E	fE'	PDAc 0.05 μM	PDAc 0.1 μM	PDAc 0.25 μM	PMA 1 μM	PMA 2.5 μM	PMA 5 μM
2nd pulse	V	E	E	E	E	E	E	E	E

Fig. 2. At concentrations of 1, 2.5, and 5 μ*M*, PMA did not potentiate E's transcriptional effect. PDAc, at concentrations of 0.05 and 0.1 μ*M* combined with E in the second pulse increased transcriptional activity by 15- and 11-fold, respectively; 0.25 μ*M* PDAC reduced the potentiating effect of E. The first three bars are from Fig. 1. $p < 0.05$ was regarded as significant. $n = 4$ per treatment group in each replicate experiment. [#]Indicates $p < 0.005$ and ^indicates $p < 0.0005$ as compared to the control group (V+V), **indicates $p < 0.0005$ as compared to E+E, [a]indicates $p < 0.0005$ and [b]indicates $p < 0.05$ as compared to fE'+E.

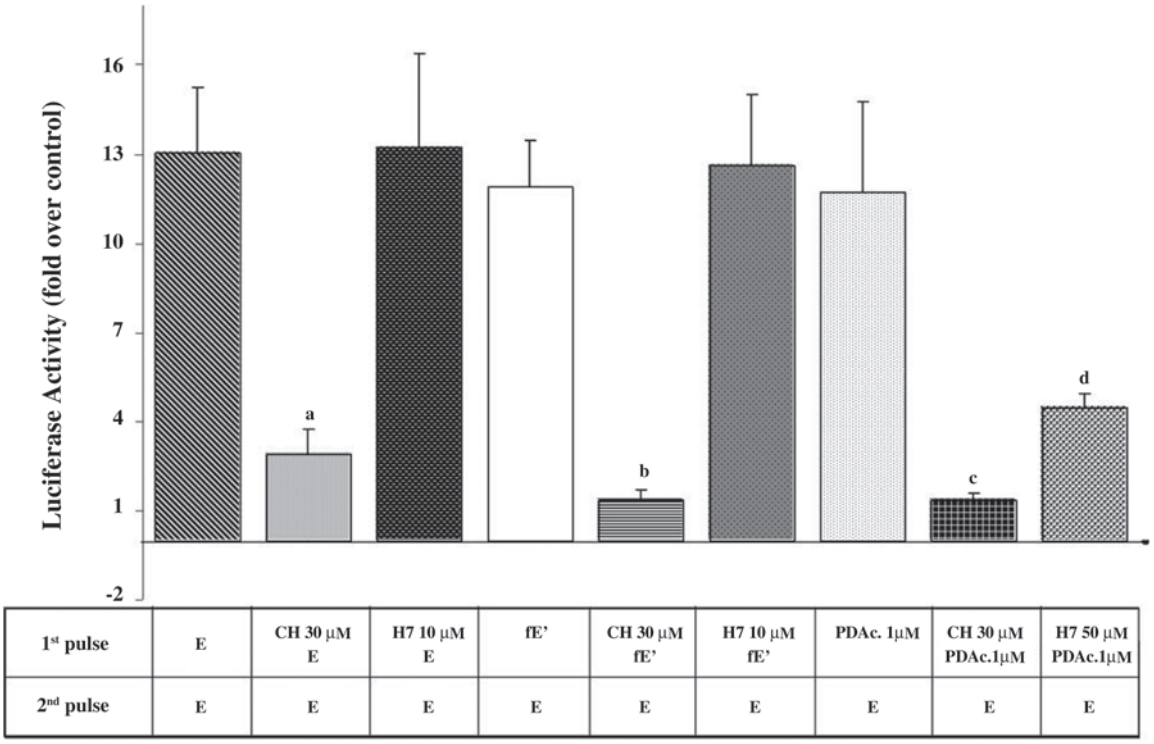
30 μ*M* CH to the E+E group blocked the transcription of the luciferase reporter gene ($p < 0.05$, CH+E+E vs E+E, Fig. 3; and $p > 0.05$, CH+E+E vs V+V, in Fig. 1). The same inhibition occurred when 30 μ*M* CH was applied to cells treated with filtered E-BSA in the first pulse and E in the second (CH+fE'+E, $p < 0.05$, compared to fE'+E group). In one group, 30 μ*M* CH was applied to cells treated with PDAc 0.1 μ*M* in the first pulse and with estradiol in the second (CH+PDAc+E). The PKC inhibitor also blocked the potentiating effect of the activator ($p < 0.005$, CH+PDAc+E vs PDAc+E, Fig. 3). Another PKC inhibitor used, H7, was less effective. It was tested in two concentrations: 10 and 50 μ*M*. At 10 μ*M*, H7 did not inhibit the groups where cells received E+E or fE'+E treatment. At 50 μ*M*, H7 attenuated PDAc 0.1 μ*M* effect ($p < 0.05$, Fig. 3).

Discussion

We have investigated possible relationships between the genomic and nongenomic actions of estradiol in the MCF-7 cell line. To show that two independent pulses of estradiol treatment can synergize, we used estradiol in one or both pulses (Fig. 1). When compared to cells treated with only the vehicle in both pulses, cells with vehicle treatment in the first pulse and estradiol treatment in second (Fig. 1, first and a last column of figure) showed 3.5-fold increase of transcriptional activity. To see whether replacement of the vehicle in the first pulse affected the outcome, we treated cells with estradiol in both pulses (Fig. 1, second column). Transcriptional activity of estradiol increased 12-fold. Our explanation for this phenomenon is that treatment with estradiol in the first pulse potentiates genomic actions of estradiol in the second pulse. Our results match with the results of other studies *(14)*, and thus prove that synergism of two pulses of estradiol is not unique to nerve cell lines.

In order to study nongenomic actions of estradiol, we used E-BSA. Although E-BSA has been shown to induce ERE-dependent transcription in MCF-7 cells, it requires an incubation of more than 1 h *(22)*. Studies done on this conjugate show that there is only a very small (<0.00063% per mL/h) rate of E-BSA dissociation *(9,23)*, which makes it impossible for E to enter the cell. This can restrict estrogen's actions to the cell membrane. In the present study, E-BSA was applied for only 15 min, a duration far shorter than that required to induce any ERE-dependent transcription (0.0001575% per mL/15 min). Therefore, the possibility that E-BSA could induce genomic action by itself can be ruled out. Replacement of E with the E-BSA in the first pulse (Fig. 1, column three), caused an 11-fold increase in transcriptional activity. Because there is no significant difference between (E+E) and (E-BSA+E) groups, we suggest that estradiol's membrane action is a strong candidate for the potentiation of the genomic actions of estradiol.

These results bring us to next logical question: What are the pathways involved in estrogen's nongenomic actions? We investigated the role of PKC similar to SK-N-BE2C



1st pulse	E	CH 30 μM E	H7 10 μM E	fE'	CH 30 μM fE'	H7 10 μM fE'	PDAc. 1μM	CH 30 μM PDAc.1μM	H7 50 μM PDAc.1μM
2nd pulse	E	E	E	E	E	E	E	E	E

Fig. 3. PKC inhibitors block E's transcription-potentiating membrane actions. Administration of 30 μ*M* CH to the E+E group blocked the transcription of the luciferase reporter gene: $p < 0.05$, CH+E+E vs E+E, Fig. 3; and $p > 0.05$ CH+E+E vs V+V in Fig. 1. The same inhibition occurred when 30 μ*M* CH was applied to cells treated with filtered E-BSA in the first pulse and E in the second CH+fE'+E, $p < 0.05$, compared with the fE'+E group. CH blocked the potentiating effect of PDAc: $p < 0.005$, CH+PDAc+E vs PDAc+E. H7 was less effective at blocking the potentiating effect. At 50 μ*M*, H7 attenuated PDAc 0.1 μ*M* effect. The means represent samples from two replicate experiments ($n = 4$ per treatment group in each replicate experiment). $p < 0.05$ was regarded as significant. The first and fourth bars represent the same as the second and third bars of Fig. 1. [a]Indicates $p < 0.05$, as compared to E+E, [c]$p < 0.0005$ as compared to PDAc. 1 μ*M*+E, [b]$p < 0.05$ as compared to fE'+E and [d]$p < 0.05$ as compared to PDAc.1 μ*M*+E.

cells, where PKC was shown to play a role in the nongenomic mechanisms of estrogen, and our results indicate that PKC is necessary and sufficient for the mediation of the potentiation by membrane actions. We further found differences in effects among the PKC activators and inhibitors. In mimicking E-BSA actions, PDAc is much more effective than PMA. But in inducing β-gal transcription, the situation was reversed (data not shown). The potentiating effect of PDAc was blocked by chelerythrine, but not by H7, whereas the β-gal–inducing action of PMA was blocked by H7 but not chelerythrine. The exact reason(s) underlying these interesting differences is not yet known. It could be that potentiating membrane actions of E-BSA are mediated by specific PKC isozyme(s) that is activated and inhibited by PDAc and chelerythrine, respectively, but not by PMA and H7. This explanation is supported by the following findings. First, different phorbol esters may activate different sets of isozymes or a similar set but with differential efficiencies. For example, phorbol 12,13-dibutyrate pretreatment caused a partial downregulation of the PKC-α and -βI and a complete downregulation of the novel δ- and ε-isozymes, but had no effect on the atypical PKC-ζ *(24)*. Second, different PKC isozymes can serve different functions in single cells *(25)*. For example, in MCF-7 cells growth is stimulated by PKC-η, but inhibited by PKC-δ *(26)*. Therefore, it is possible that one isozyme mediates estradiol's membrane actions while another one stimulates β-gal transcription. Third, different ligands, including hormones, can activate specific PKC isozymes *(25)*. For instance, 1,25-$(OH)_2D_3$ increased PKC-α, but not PKC-βI, -ε, or -ζ, levels in the nuclear fraction in a time-dependent manner *(27)*. Parathyroid hormone activates specific PKC isozymes (α and β) *(28)*. Even E, with chronic treatment, affects specific isozymes—it upregulates PKC-δ in the rat and rabbit corpus luteum and luteinized rat granulosa primary cell culture, but downregulates the same isoform in MCF-7 cells *(29)*. Similarly, tamoxifen stimulates PKC-ε, but not -α, -β, -γ, -δ, or -ξ in MCF-7 cells *(30)*. This explanation can be evaluated with more isozyme-specific antagonists. Estrogen was found to activate PKC in a nongenomic manner in female rat cells; and chelerythrine, a specific inhibitor of PKC, inhibits E-dependent alkaline phosphatase activity and proteoglycan sulfation in these cells, indicating PKC is involved in the signal transduction mechanism for E's membrane actions *(31)*.

A large fraction of breast cancer cases are associated with the activation of estrogen receptors *(32–34)* and/or PKC *(35–38)* in breast tissues. Therefore, our findings that, in

a breast cancer cell line, estrogen's membrane-initiated actions led to the potentiation of estrogen's genomic actions and that this potentiation involved the activation of PKC, are biologically relevant, especially to the pathophysiology of the breast cancer. This synergism between membrane-initiated and genomic actions of estrogen has also been proven to be biologically relevant in behaviors of whole animals *(37)*. Furthermore, the findings not only are biologically relevant but can also serve as platforms to investigate the mechanisms underlying estrogens' membrane-initiated actions, to find out how PKC is activated, to characterize the membrane estrogen receptors involved, and so on. With such investigations we would gain knowledge relevant to breast cancer and to normal endocrine physiology.

Materials and Methods

Materials

Water soluble 17β-estradiol (cyclodextrin-encapsulated) that has been used successfully on breast cancer cells by others *(39)* was employed. It was dissolved in Dulbecco's phosphate-buffered saline (D-PBS) to make 1 m*M* stock solution, and then further diluted with D-PBS to appropriate concentrations, as is shown in the Results. Another estrogen, 17β-estradiol (E) conjugated to BSA (E-BSA or E') [β-estradiol 6-(*O*-carboxymethyl)oxime: BSA], was used in order to prevent estradiol from entering cells. E-BSA was first dissolved in Tris-buffered (pH 8) distilled water to make 1 m*M* stock solution and then diluted with D-PBS to 10^{-9} *M* for the experiments. To ensure the absence of free estradiol, E-BSA was filtered using a procedure that successfully removes free estradiol *(9)*. This filtered E-BSA (fE') was used throughout the study. PKC inhibitors, chelerythrine chloride (1,2-dimethoxy-*N*-methyl [1,3] benzodioxolo [5,6-c] phenanthridinium chloride) and H7-dihydrochloride: (1-[5-isoquinolinesulfonyl]-2-methylpiperazine) and activators, PMA (phorbol 12-myristate 13-acetate) and PDAc (phorbol 12,13-diacetate) were dissolved in double-distilled water (ddH2O) or dimethylsulfoxide (DMSO) to make stock solutions of 10 m*M*. They were diluted with hormone free (H-free) media to appropriate concentrations. All reagents were purchased from Sigma (St. Louis, MO, USA).

Cell Culture

MCF-7 human breast cancer cells were obtained from American Type Culture Collection (Manassas, VA). They were maintained in Dulbecco's modified Eagle's medium (DMEM) and F-12 nutrient mixture (HAM) (1:1), which contained 10% fetal bovine serum (FBS) (Bioreclamation, New York), 100 units/mL penicillin and 50 μg/mL streptomycin. They were incubated at 37°C in a humidified incubator containing 95% air and 5% CO_2.

During the experiments, phenol red-free HAM and charcoal-stripped FBS (Gemini Biotech, Alachua, FL) were used in order to remove all possible estrogenic actions from the culture medium.

Plasmid Construct

Two plasmids were employed. The PGL2-TATA-Inr-luc construct (5687 bp), which has three consensus estrogen-response elements (EREs) in tandem at 37Bgl II upstream of the luciferase reporter gene, was used for monitoring the genomic transcriptional action of estradiol. This 3xERELuc construct is a generous gift from Dr. Donald McDonnell. For monitoring and controlling for transfection efficiencies, the pSV-β-galactosidase (β-gal) construct (6821 bp) with an early promoter SV40 was used.

Transfection

MCF-7 human breast cancer cells were plated in 12-well plates (Falcon) at a density of 0.2 × 106 cells per well. For transfection, the Effectene Reagent Kit (Qiagen) was used following manufacturer's instructions. After reaching 60–70% confluence, cells were transfected with 3xERELuc construct (150 ng/μL) and β-Gal Plasmid (Promega) (150 ng/μL). Cells were incubated for 24 h.

Treatment

Transfected cells were treated with the two-pulse paradigm mentioned in the Introduction that consists of two treatment pulses separated by 4 h. E2 (10^{-11}–10^{-8} *M*), E-BSA (10^{-9} *M*), and other test agents were used in various combinations as indicated in the Results. After each treatment pulse, cells were washed with D-PBS and then incubated in fresh, phenol red-free media. After overnight incubation, they were washed twice with D-PBS and lysed by adding 200 μL of Reporter Lyses Buffer (Promega) containing 1 mg/mL BSA. Lysate was collected and used for luciferase (using Luciferase Assay Reagent from Promega) and β-Gal (1xRLB+2xβ-gal assays, 50 mL of which contained: 5 mL K_2HPO_4 (1 *M*), 5 mL KH_2PO_4 (1 *M*), 350 μL 2-mercaptoethanol (Bio-Rad), 40 μL $MgCl_2$ (5 *M*), 66.5 mg *o*-nitrophenyl-β-D-galactopyranoside, autoclaved distilled water, volume to 50 mL. Both assays were performed according to the manufacturer's protocols (Promega). In order to control for the transfection efficiency, luciferase expression was normalized against β-gal expression (value of luciferase expression/value of β-gal expression). For comparisons across experiments, results of experimental groups are expressed in terms of multiples of the control groups' results.

Controls

Previously in vitro (SK-N-BE2C human neuroblastoma cells) and in vivo (lordosis behavior in rats) studies investigating genomic and membrane actions of estrogens were done in our laboratory *(37,38)*. In this current research our main interest was simply to determine whether the synergism between membrane and genomic actions can also occur in non-neural cells. The methods used here are adapted from and hence are very similar to those in the above-mentioned studies. In those studies the controls (a) looking for effects of E-BSA alone in either pulse, (b) reversing the

order of pulses, and (c) the effects of activators and inhibitors without any E were all performed successfully and did not need to be repeated.

Statistical Analyses

For comparisons among experimental groups, a one-way ANOVA was used, followed by Turkey post-hoc test (Statistica, StatSoft, Tulsa, OK). $p < 0.05$ was regarded as significant. Numbers of wells per group was always ≥ 8.

Acknowledgments

We would like to thank Dr. J.A. Mong and Dr. N. Vasudevan for their advice and assistance. This work was supported by grant HD 05751.

References

1. Aranda, A. and Pascual, A. (2001). *Physiol. Rev.* **81,** 1269–1304.
2. Komm, B. S., Terpening, C. M., Benz, D. J., et al. (1988). *Science* **241,** 81–84.
3. Luconi, M., Forti, G., and Baldi, E. (2002). *J. Steroid Biochem. Mol. Biol.* **80,** 369–381.
4. Mendelsohn, M. E. (2002). *Am. J. Cardiol.* **89,** 12E–17E.
5. Kuiper, G. G., Carlsson, B., Grandien, K., et al. (1997). *Endocrinology* **138,** 863–870.
6. Truss, M. and Beato, M. (1993). *Endocr. Rev.* **14,** 459–479.
7. Aronica, S. M., Kraus, W. L., and Katzenellenbogen, B. S. (1994). *Proc. Natl. Acad. Sci. USA* **91,** 8517–8521.
8. Le Mellay, V., Grosse, B., and Lieberherr, M. (1997). *J. Biol. Chem.* **272,** 11902–11907.
9. Stevis, P. E., Deecher, D. C., Suhadolnik, L., Mallis, L. M., and Frail, D. E. (1999). *Endocrinology* **140,** 5455–5458.
10. Tesarik, J. and Mendoza, C. (1995). *J. Clin. Endocrinol. Metab*. **80,** 1438–1443.
11. Pietras, R. J. and Szego, C. M. (1977). *Nature* **265,** 69–72.
12. Razandi, M., Pedram, A., Greene, G. L., and Levin, E. R. (1999). *Mol. Endocrinol.* **13,** 307–319.
13. Wyckoff, M. H., Chambliss, K. L., Mineo, C., et al. (2001). *J. Biol. Chem.* **276,** 27071–27076.
14. Vasudevan, N., Kow, L. M., and Pfaff, D. W. (2001). *Proc. Natl. Acad. Sci. USA* **98,** 12267–12271.
15. Newton, A. C. and Johnson, J. E. (1998). *Biochim. Biophys. Acta* **1376,** 155–172.
16. Mochly-Rosen, D. and Kauvar, L. M. (1998). *Adv. Pharmacol.* **44,** 91–145.
17. Basu, A. (1998). *Mol. Pharmacol.* **53,** 105–111.
18. Blobe, G. C., Sachs, C. W., Khan, W. A., et al. (1993). *J. Biol. Chem.* **268,** 658–664.
19. Signorelli, P., Luberto, C., and Hannun, Y. A. (2001). *FASEB J.* **15,** 2401–2414.
20. Sylvia, V. L., Walton, J., Lopez, D., Dean, D. D., Boyan, B. D., and Schwartz, Z. (2001). *J. Cell Biochem.* **81,** 413–429.
21. Power, K. A. and Thompson, L. U. (2003). *Breast Cancer Res. Treat.* **81,** 209–221.
22. Seo, H. S. and Leclercq, G. (2002). *J. Steroid Biochem. Mol. Biol.* **80,** 109–123.
23. Caldwell, J. D. and Moe, B. D. (1999). *Horm. Behav.* **35,** 38–46.
24. Rivera-Bermudez, M. A., Bertics, P. J., Albrecht, R. M., Mosavin, R., and Mellon, W. S. (2002). *Mol. Cell Endocrinol.* **188,** 227–239.
25. Jaken, S. (1996). *Curr. Opin. Cell Biol.* **8,** 168–173.
26. Fima, E., Shtutman, M., Libros, P., et al. (2001). *Oncogene* **20,** 6794–6804.
27. Sanders, J. L. and Stern, P. H. (2000). *J. Bone Miner. Res.* **15,** 885–893.
28. Pickett, C. A., Manning, N., Akita, Y., and Gutierrez-Hartmann, A. (2002). *Mol. Endocrinol.* **16,** 2840–2852.
29. Shanmugam, M., Krett, N. L., Maizels, E. T., et al. (1999). *Mol. Cell Endocrinol.* **148,** 109–118.
30. Lavie, Y., Zhang, Z. C., Cao, H. T., et al. (1998). *Int. J. Cancer* **77,** 928–932.
31. Improta-Brears, T., Whorton, A. R., Codazzi, F., York, J. D., Meyer, T., and McDonnell, D. P. (1999). *Proc. Natl. Acad. Sci. USA* **96,** 4686–4691.
32. Lauber, A. H., Mobbs, C. V., Muramatsu, M., and Pfaff, D. W. (1991). *Endocrinology* **129,** 3180–3186.
33. Parsons, B., McEwen, B. S., and Pfaff, D. W. (1982). *Endocrinology* **110,** 613–619.
34. Powell, C. E., Soto, A. M., and Sonnenschein, C. (2001). *J. Steroid Biochem. Mol. Biol.* **77,** 97–108.
35. Kow, L. M. and Pfaff, D. W. (1998). *Behav. Brain Res.* **92,** 169–180.
36. Kow, L. M., Vasudevan, N., Devidze, N., Ragnauth, A., and Pfaff, D. W. (2005). In: Kordon, C., Gaillard, R. C., and Christen, Y. (eds.). *Hormones and the brain.* Springer-Verlag: Berlin.
37. Kow, L. M. and Pfaff, D. W. (2004). *Proc. Natl. Acad. Sci. USA* **101,** 12354–12357.
38. Vasudevan, N., Kow, L. M., and Pfaff, D. W. (2001). *Proc. Natl. Acad. Sci. USA* **98,** 12267–12271.
39. Filardo, E. J., Quinn, J. A., Bland, K. I., and Frackelton, A. R. Jr. (2000). *Mol. Endocrinol.* **14,** 1649–1660.