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Cross-talk between the estrogen receptor-, protein kinase A-, and mitogen-activated protein kinase-mediated signaling pathways in the regulation of lactotroph proliferation in primary culture

Koji Yamakawa, Jun Arita*

Department of Physiology, University of Yamanashi Interdisciplinary Graduate School of Medicine and Engineering, Tamaho, Nakakoma, Yamanashi 409-3898, Japan

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Abstract

Using pharmacological means we investigated the functional interactions between the estrogen receptor (ER)-, protein kinase A (PKA)-, and mitogen-activating protein kinase (MAPK)-mediated pathways in the regulation of lactotroph proliferation in primary culture. Treatment of lactotrophs for 28 h with the PKA inhibitor H89 or KT5720, an effective inhibitor of forskolin-induced proliferation, inhibited both insulin- and estradiol-induced proliferation. Inhibition of the MAPK activity by PD98059 or U0126 suppressed not only insulin-induced proliferation but also forskolin- and estradiol-induced proliferation. However, treatment for 28 h with the antiestrogens 4-hydroxy tamoxifen and ICI182780 failed to antagonize estradiol-induced lactotroph proliferation but instead enhanced it. Prolonging the antiestrogen treatment time from 28 to 88 h was effective in antagonizing estradiol-induced proliferation with this long-term treatment also inhibiting insulin- and forskolin-induced proliferation. There was no decrease in these mitogen-induced proliferations following treatment with a progesterone antagonist or protein kinase C inhibitor. These results suggest that cross-talk occurs between the ER-, PKA-, and MAPK-mediated signaling pathways in the regulation of lactotroph proliferation, and that antiestrogens stimulate and inhibit estradiol-induced proliferation in a time-dependent manner.

Keywords: Estradiol; Antiestrogen; Mitogen-activated protein kinase; Protein kinase A; Estrogen receptor; Lactotroph; Proliferation; Cross-talk

1. Introduction

Proliferation of lactotrophs in the anterior pituitary gland is regulated by multiple extracellular signals that originate from distinct tissues. Firstly, estradiol secreted from the ovaries stimulates lactotroph proliferation via a direct action [1–4], in addition to an indirect action on the hypothalamus [5–7]. The direct mitogenic action is mediated by estrogen receptors (ERs) with the mechanism of this action yet to be fully understood [8,9]. Secondly, hypothalamic hormones, the secretion of which may be modified by estradiol, act on lactotrophs via hypophysial portal blood and are able to stimulate or inhibit cell proliferation [10–12]. Dopamine and its agonist, bromocriptine, markedly inhibit lactotroph proliferation [13–15]. This dopaminergic inhibition is mediated partially by decreased synthesis of intracellular cyclic AMP, which serves as a positive intracellular regulator of lactotroph proliferation by activating protein kinase A (PKA) [16]. Thirdly, a number of growth factors released intrinsically by the anterior pituitary act in an autocrine or paracrine manner [17] to modify lactotroph proliferation [18]. We have also shown previously that insulin acting through insulin-like growth factor (IGF)-I receptors stimulates lactotroph proliferation via the mitogen-activated protein kinase (MAPK) cascade [16].

Cell proliferation in estrogen-responsive organs, such as the uterus, mammary gland, and anterior pituitary gland, is a process regulated by complex interactions between a number of intracellular signal transduction pathways. For example, profound interactions between the estrogen/ER- and growth factor/MAPK-mediated signaling pathways have been shown by numerous findings of modifications of growth factor production and signaling by estrogens [19] and those of ER signaling by growth factors [20]. In addition, blockade of the estrogen/ER signaling pathway affects the mitogenic action of growth factor while blockade of the growth factor/MAPK pathway affects the mitogenic action of estrogen [21]. However, little is known about interactions between cyclic AMP/PKA- and estrogen/ER-

* Corresponding author. Tel.: +81-55-273-6730; fax: +81-55-273-6730.
E-mail address: jarita@yamanashi.ac.jp (J. Arita).

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or growth factor/MAPK-mediated signalings in the regulation of cell proliferation in estrogen-responsive tissues. We have shown previously in lactotrophs that insulin-induced, MAPK-dependent proliferation is suppressed by PKA inhibitors and that forskolin or dibutyryl cyclic AMP-induced, PKA-dependent proliferation is reduced by inhibiting MEK1/2 that is located immediately upstream of MAPK [16].

The present study was therefore undertaken to elucidate the interactions between the estrogen/ER-, cyclic AMP/PKA-, and growth factor/MAPK-mediated signaling pathways in the regulation of cell proliferation. We examined systematically the interactions in rat lactotroph proliferation grown in serum-free cultures. This was achieved by blocking a single pathway using pharmacological agents and observing proliferation mediated by the other pathways. There are three advantages of using lactotrophs in primary culture as an experimental model for studying interaction between pathways. Firstly, as a consequence of the difficulties of demonstrating mitogenic actions of estrogens in primary culture of uterus and mammary tissues, the majority of in vitro studies investigating interactions have used estrogen-responsive cell lines. However, the proliferation and regulation of these cell lines may not be representative of the normal physiology of cell proliferation. In contrast, lactotrophs in primary culture consistently show stimulation of cell proliferation in response to estrogens [2,3,22]. Secondly, because cyclic AMP is a negative, rather than a positive intracellular regulator of cell proliferation in many cell types [23], the lactotroph provides a good model for studying interactions between the three signaling pathways as it is the sole estrogen-responsive cell in which cyclic AMP serves as a positive regulator. Thirdly, in the same lactotroph, the interactions between the MAPK pathway and the cyclic AMP/PKA or estrogen/ER pathway have been demonstrated in the regulation of prolactin gene expression by several studies using inhibitors of signal transduction [24,25].

2. Materials and methods

2.1. Reagents

Estradiol, insulin, forskolin, 4-hydroxy tamoxifen (OHT), calphostin C, and RU486 were purchased from Sigma (St. Louis, MO, USA); U0126 was obtained from Promega (Madison, WI, USA); PD98059 and KT5720 from Biomol Research Laboratories (Plymouth Meeting, PA, USA); H89 was purchased from Seikagaku (Tokyo, Japan) and ICI182780 from Tocris (Ballwin, MO, USA).

2.2. Cell culture

The experiments were conducted under the guidelines of the Ethical Committee of Animal Experiments of University of Yamanashi. Six weeks old female Wistar rats purchased from Japan SLC (Shizuoka, Japan) were used to obtain the anterior pituitary cells for primary culture. The cells were dispersed as described previously [22]. Briefly, anterior pituitaries were minced in minimum essential medium for suspension (Sigma) containing $NaHCO_3$, penicillin G streptomycin, bovine serum albumin (BSA), and HEPES (S-MBH). The mixture was incubated with constant stirring at 37 °C for 90 min in S-MBH containing 0.01% trypsin and 0.005% DNase in a siliconized spinner suspension flask. The dispersed pituitary cells were treated with soybean trypsin inhibitor and DNase followed by a cell count and a viability test. A 100 μl aliquot of cell suspension containing 1.2×10^5 cells in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mix F12 without phenol red, containing HEPES, penicillin, and streptomycin (DMEM/F12) (Sigma) was placed on poly-D-lysine-coated 35 mm culture dishes (Falcon, Becton Dickinson, Bedford, MA, USA). The pituitary cells were incubated at 37 °C for 60 min in a humidified CO_2 incubator and then flooded with 2 ml DMEM/F12 containing 150 ng/ml insulin.

After pre-culture for 1 day, the pituitary cells were washed with DMEM/F12 and used for the series of experiments. Cultures for experiments were initiated with a serum-free, chemically defined medium [22] that was replaced fresh every 2 days during the culture period. Cells were treated with one of the mitogens, estradiol, insulin, or forskolin for 88 h. In the majority of experiments, an inhibitor of each signaling pathway was added for the last 28 h of mitogen treatment (Fig. 1). For labeling proliferating pituitary cells, 200 μM 5-bromo-2′-deoxyuridine (BrdU) (Sigma) was added 18 h prior to the end of the culture. At the end of culture, the pituitary cells were redispersed with trypsin, detached from the culture dishes, suspended in Hanks' balanced salt solution containing HEPES, and attached to poly-D-lysine-coated glass slides by centrifugation with a cytocentrifuge (SC-2, Tomy, Tokyo, Japan). The cells attached on glass slides were fixed in ice-cold methanol for 30 min and stored in phosphate-buffered saline (PBS) at 4 °C until immunostaining for prolactin (PRL) and BrdU.

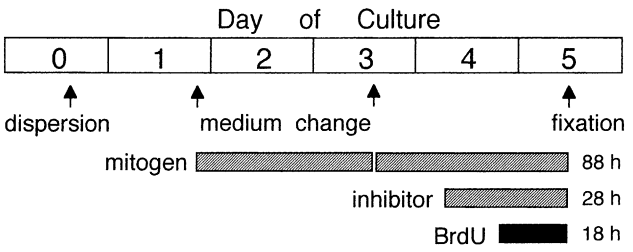

Fig. 1. The treatment schedule used to examine the effects of inhibitors of intracellular signaling pathways on mitogen-induced proliferation of lactotrophs. Anterior pituitary cells in primary culture were treated with one of the mitogens, estradiol, insulin, or forskolin for 88 h, with an inhibitor of the ER-, MAPK-, or PKA-mediated signaling pathway for the final 28 h, and with BrdU for the final 18 h of the culture. In some of the experiments on antiestrogens, the treatment time of the inhibitors was changed from 28 h.

2.3. Immunostaining

The anterior pituitary cells attached to the glass slides were double immunostained for BrdU and PRL, as described previously [7]. Briefly, the slides were treated with 3 M HCl for 30 min, neutralized with 0.1 M borate buffer, pH 8.5 for 10 min, and then blocked with 10% normal donkey serum in PBS for 20 min. Double-labeling immunofluorescence staining was performed in three steps using the following reagents: (1) a mixture of a mouse monoclonal anti-BrdU antibody (Sigma) at a 1:200 dilution and a rabbit anti-rat PRL antibody (NIDDK IC-5) at a 1:4000 dilution; (2) a horse biotinylated anti-mouse IgG antibody (Vector, Burliname, CA, USA) at a 1:50 dilution; and (3) a mixture of Texas Red-labeled streptavidin (Amersham, Arlington Heights, IL, USA) at a 1:50 dilution and a donkey fluorescein-isothiocyanate (FITC)-labeled anti-rabbit IgG antibody (Amersham) at a 1:50 dilution. The cells were incubated with 70 μl of each reagent diluted with PBS containing 10% normal donkey serum for 1 h followed by a 20 min wash. The immunostained slides were covered with PermaFluor (Immunon, Pittsburgh, PA, USA) and examined using a fluorescence microscope (BX50-FLA, Olympus, Tokyo, Japan) equipped with a dual band mirror unit for FITC and Texas Red (U-DM-FI/TX).

2.4. Statistical analysis

A total of 1000 PRL-immunoreactive cells were examined in randomly chosen fields for each slide in order to determine the BrdU-labeling index, calculated as the percentage of pituitary cells that stained for both PRL and BrdU relative to the total number of PRL-immunoreactive cells. Three slides were analyzed for each treatment group with the experiments being replicated at least three times using separate batches of cell preparations. Differences between groups were analyzed statistically using one-way analysis of variance followed by Bonferroni's test.

3. Results

3.1. Inhibition of estradiol-, forskolin-, or insulin-induced lactotroph proliferation by treatment with protein kinase A inhibitors

Insulin, a potent growth factor used in numerous studies to supplement serum-free culture media, was selected as the mitogen to activate the MAPK cascade, due to this response that follows its binding to IGF-I receptors. Forskolin was used to stimulate adenylate cyclase directly, leading to increased intracellular concentrations of cyclic AMP and thereby activation of PKA. The mitogens, estradiol, insulin, and forskolin, were used at concentrations of 1 nM, 50 ng/ml, and 1 μM, respectively, comparable to those reported in previous studies [26]. As illustrated in Fig. 1, anterior pituitary

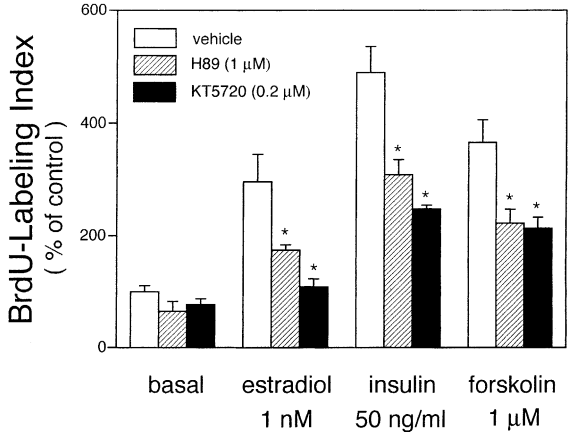

Fig. 2. Inhibition of estradiol-, insulin-, and forskolin-induced lactotroph proliferation by treatment with protein kinase A inhibitors. Anterior pituitary cells in primary culture were treated with or without 1 nM estradiol, 50 ng/ml insulin or 1 μM forskolin for 88 h in the presence of vehicle or the PKA inhibitor H89 at 1 μM or KT5720 at 0.2 μM for the final 28 h of the culture. Lactotroph proliferation was determined by BrdU-labeling for 18 h, with the BrdU-labeling index expressed relative to the group treated with no mitogen or inhibitor. Data are the mean ± S.E.M. of triplicate determinations from a representative experiment. (*) Significant difference compared to the corresponding vehicle-treated group.

cells in primary culture were treated with one of these mitogens for 88 h or alternatively with no mitogen in order to determine basal proliferation. Inhibitors of each signaling pathway investigated were added for 28 h before the end of mitogen treatment. Based on the results from preliminary experiments, 1 μM H89 and 0.2 μM KT5720 were chosen as the optimal concentrations required to block PKA activity. This concentration of H89 was considerably lower than that reported to nonspecifically inhibit other protein kinases such as cyclic GMP-dependent protein kinase [27]. As shown in Fig. 2, these concentrations were effective in inhibiting a 3.7-fold increase in lactotroph proliferation induced by treatment with 1 μM forskolin ($P < 0.05$). Treatment with estradiol or insulin significantly raised basal proliferative levels 3.0- or 4.9-fold, respectively ($P < 0.05$). While both PKA inhibitors did not influence lactotroph proliferation under basal conditions, they inhibited mitogen-induced proliferation. For example, estradiol-induced proliferation was lowered to 59 and 37% by H89 and KT5720, respectively, while insulin-induced proliferation decreased to 63 and 50%, respectively ($P < 0.05$).

3.2. Inhibition of estradiol-, forskolin or insulin-induced lactotroph proliferation by treatment with MEK1/2 inhibitors

In order to block the MAPK cascade, the MEK1/2 inhibitors PD98059 and U0126 were used at concentrations of 50 and 1 μM, respectively. These concentrations were effective in reducing insulin-induced lactotroph proliferation to 60–67% ($P < 0.05$), a finding that confirms the ability of these agents to block MAPK activity (Fig. 3). Treatment

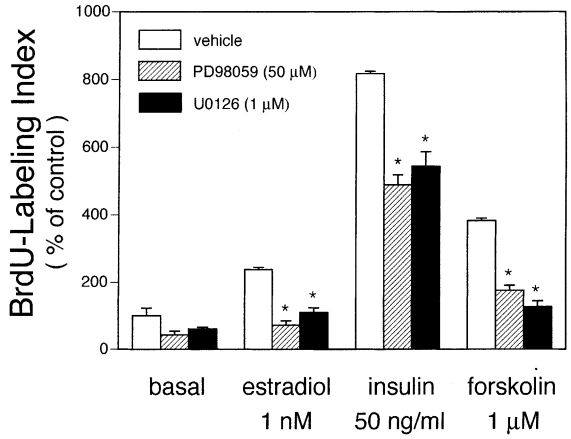

Fig. 3. Inhibition of estradiol-, insulin-, or forskolin-induced lactotroph proliferation by treatment with MEK1/2 inhibitors. Anterior pituitary cells were treated with or without 1 nM estradiol, 50 ng/ml insulin or 1 μM forskolin for 88 h in the presence of vehicle or the MEK1/2 inhibitor PD98059 at 50 μM or U0126 at 1 μM for the final 28 h of the culture. Lactotroph proliferation was determined by BrdU-labeling for 18 h, with the BrdU-labeling index expressed relative to the group treated with no mitogen or inhibitor. Data are the mean ± S.E.M. of triplicate determinations from a representative experiment. (*) Significant difference compared to the corresponding vehicle-treated group.

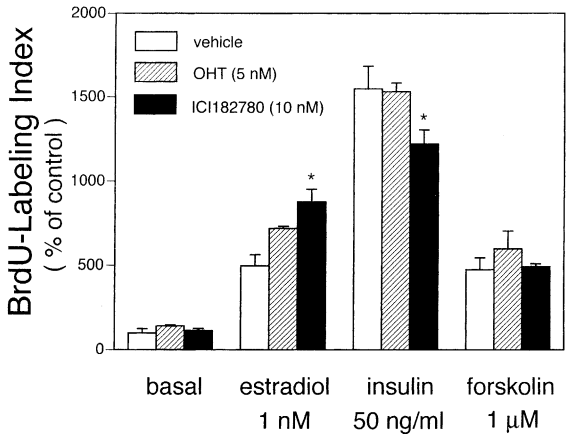

Fig. 4. Effects on estradiol-, insulin-, or forskolin-induced lactotroph proliferation of treatment with antiestrogens for 28 h. Anterior pituitary cells were treated with or without 1 nM estradiol, 50 ng/ml insulin or 1 μM forskolin for 88 h in the presence of vehicle or the antiestrogen OHT at 5 nM or ICI182780 at 10 nM for the final 28 h of the culture. Lactotroph proliferation was determined by BrdU-labeling for 18 h, with the BrdU-labeling index expressed relative to the group treated with no mitogen or inhibitor. Data are the mean ± S.E.M. of triplicate determinations from a representative experiment. (*) Significant difference compared to the corresponding vehicle-treated group.

with PD98059 and U0126 for 28 h significantly decreased the levels of proliferation induced by estradiol to 30 and 46%, respectively, and those induced by forskolin to 46 and 33%, respectively ($P < 0.05$).

3.3. Effects of treatment with estrogen antagonists on estradiol-, forskolin- or insulin-induced lactotroph proliferation

Based on the results of our previous study [26], 10 nM ICI182780, a pure estrogen antagonist, and 5 nM OHT, an active metabolite of the mixed antagonist/agonist tamoxifen, were used to block ER function. The concentration of ICI182780 was lower than the concentration at which it inhibits progesterone-induced gene transcription [28]. Whilst attempting to ensure the effectiveness of the antiestrogens at these concentrations, we observed no antagonism associated with this treatment performed over the last 28 h of the 88 h estradiol treatment period (Fig. 4). Rather than inhibiting lactotroph proliferation induced by estradiol, ICI182780 and OHT both caused a 76% ($P < 0.05$) and 45% increase in proliferation, respectively, although the effect of OHT was not statistically significant. Antiestrogen treatment did not alter either basal proliferation or insulin- and forskolin-induced proliferation with the exception of a significant decrease in insulin-induced proliferation with ICI182780 ($P < 0.05$). The inability of the antiestrogens to antagonize the estrogen mitogenic action led us to examine the time course of their antagonistic actions. Anterior pituitary cells treated with estradiol for 88 h were exposed to ICI182780 or OHT for varying times before the end of the culture. The significant enhancement of estradiol-induced proliferation observed after 28 h of antiestrogen treatment was abolished by prolonging the treatment time from 28 to 52 h (i.e., advancing the initiation of the treatment by 24 h). In contrast, estradiol-induced proliferation was prevented by longer periods of antiestrogen treatment ($P < 0.05$) (Fig. 5). Antiestrogen treatment for the entire period of

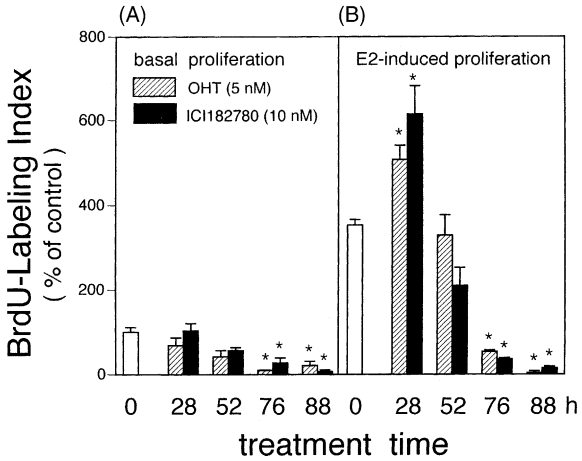

Fig. 5. Time-course for the effects of antiestrogens on (A) basal and (B) estradiol-induced proliferation of lactotrophs. Anterior pituitary cells were treated with 1 nM estradiol (B) or without 1 nM estradiol (A) for 88 h in the presence of vehicle or the antiestrogen OHT at 5 nM or ICI182780 at 10 nM for varying times before the end of culture. Lactotroph proliferation was determined by BrdU-labeling for 18 h, with the BrdU-labeling index expressed relative to the group treated with no estradiol or antiestrogen. Data are the mean ± S.E.M. of triplicate determinations from a representative experiment. (*) Significant difference compared to the corresponding vehicle-treated group.

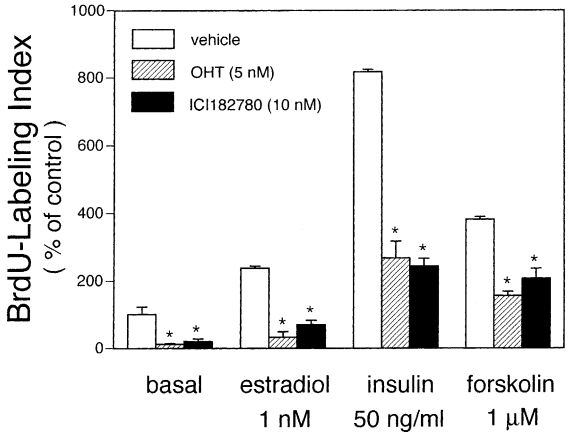

Fig. 6. Effects on estradiol-, insulin-, or forskolin-induced lactotroph proliferation of treatment with antiestrogens for 88 h. Anterior pituitary cells were treated with or without 1 nM estradiol, 50 ng/ml insulin or 1 μM forskolin for 88 h in the presence of vehicle or the antiestrogen OHT at 5 nM or ICI182780 at 10 nM for 88 h. Lactotroph proliferation was determined by BrdU-labeling for 18 h, with the BrdU-labeling index expressed relative to the group treated with no mitogen or inhibitor. Data are the mean ± S.E.M. of triplicate determinations from a representative experiment. (*) Significant difference compared to the corresponding vehicle-treated group.

estradiol treatment caused near complete inhibition of both basal and estradiol-induced proliferation.

Based on the results of the time course study, we examined the effects of antiestrogen treatment for 88 h on insulin- and forskolin-induced lactotroph proliferation. Long-term administration of OHT or ICI182780 resulted in 33 and 30% reduction in insulin-induced proliferation, respectively ($P < 0.05$) (Fig. 6). Similarly, the antiestrogens were effective in inhibiting the increase in forskolin-induced proliferation ($P < 0.05$).

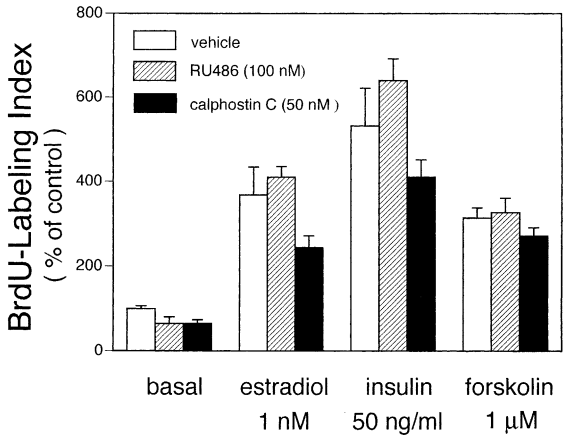

Fig. 7. Effects on estradiol-, insulin-, or forskolin-induced lactotroph proliferation of treatment with a progesterone antagonist or a protein kinase C inhibitor. Anterior pituitary cells were treated with or without 1 nM estradiol, 50 ng/ml insulin or 1 μM forskolin for 88 h in the presence of vehicle, the progesterone antagonist RU486 at 100 nM, or the protein kinase C inhibitor calphostin C at 50 nM for the final 28 h of the culture. Lactotroph proliferation was determined by BrdU-labeling for 18 h, with the BrdU-labeling index expressed relative to the group treated with no mitogen or inhibitor. Data are the mean ± S.E.M. of triplicate determinations from a representative experiment.

3.4. Effects of treatment with a progesterone antagonist or a protein kinase C (PKC) inhibitor on estradiol-, forskolin- or insulin-induced lactotroph proliferation

The effects of blockade of other signaling pathways related to ERs and PKA were also examined. Based on the results reported in earlier studies [29,30], we selected 100 nM RU486 and 50 nM calphostin C as suitable concentrations to block the progesterone receptor- and PKC-mediated signaling pathways, respectively. Treatment with RU486 and calphostin C at these concentrations did not significantly alter lactotroph proliferation induced by estradiol, insulin, or forskolin ($P > 0.05$) (Fig. 7).

4. Discussion

This study clearly demonstrates interactions between cyclic AMP/PKA- and estrogen/ER-mediated signaling in the regulation of lactotroph proliferation. Treatment with PKA inhibitors for 28 h inhibited estradiol-induced proliferation, while treatment with antiestrogens for 88 h, but not 28 h, inhibited forskolin-induced proliferation. These are the first results in estrogen-responsive tissues including the lactotroph to demonstrate that PKA and ER activity are required for the mitogenic actions of estrogen and cyclic AMP, respectively. To date, the functional interactions between the cyclic AMP/PKA and estrogen/ER signaling pathways have been restricted to gene transcription. Aronica and Katzenellenbogen [31] demonstrated that in addition to estradiol, cyclic AMP-increasing agents stimulated ER-activated gene transcription in cultured uterine cells, and that transcriptional activation by estradiol and cyclic AMP-increasing agents was suppressed by treatment with an inhibitor panspecific for cyclic nucleotide-dependent protein kinases and an antiestrogen, respectively. The suppression of cyclic AMP-mediated activation by antiestrogens has also been shown in ER-mediated transcription in HeLa cells [32] and MCF-7 breast cancer cells [33]. Our study extends these findings on gene transcription by demonstrating an interaction between the cyclic AMP/PKA and estrogen/ER-mediated signaling pathways involved in cell proliferation. However, the mechanisms by which PKA inhibitors and antiestrogens inhibited estradiol- and forskolin-induced lactotroph proliferation, respectively, remain to be clarified. There are several lines of evidence suggesting that inhibition of estradiol-induced proliferation by PKA inhibitors may be due, in part, to blocking the increased intracellular cyclic AMP levels induced by estrogen. Firstly, treatment with estrogen stimulates adenylate cyclase activity thereby increasing intracellular levels of cyclic AMP in a variety of tissues [34–37]. Secondly, recent studies have shown the existence of membrane ERs that

activate second messenger-generating cascades, including adenylate cyclase, to exert a nongenomic action [36,38,39]. Thirdly, it is possible that such a nongenomic action rather than a genomic action is involved in the mitogenic action of estradiol [36,40,41]. On the other hand, inhibition of forskolin-induced proliferation by antiestrogens may be attributable in part to the ability of cyclic AMP/PKA to increase ER-mediated transcriptional activation in an estrogen-independent manner [31,32] or by acting synergistically with estrogen [42,43]. Furthermore, PKA has been shown to phosphorylate ER at serine 236 in the domain containing the transcription activation function-1 [31,44,45]. While the relationship between PKA-induced ER phosphorylation and transcriptional activation, however, remains poorly understood, it is possible that inhibition by antiestrogens of PKA transcriptional activation and/or ER phosphorylation may lead to suppression of forskolin-induced cell proliferation.

The present study demonstrated inhibition of insulin- and forskolin-induced lactotroph proliferation by PKA inhibitors and MEK1/2 inhibitors, respectively. These results indicate that interactions occur between the cyclic AMP/PKA- and insulin/MAPK-mediated signaling pathways and confirm the results of our previous studies [16]. To account for these interactions, we hypothesize that a convergence molecule exists that requires signals from both the cyclic AMP/PKA- and insulin/MAPK-mediated pathways in order to stimulate proliferation. However, the inhibition of insulin-induced proliferation by PKA inhibitors may be partially due to the ability of these inhibitors to block nuclear translocation of MAPK [46,47].

We also demonstrated inhibition of estradiol-induced proliferation with MEK1/2 inhibitors and insulin-induced proliferation following 88 h treatment with antiestrogens. These results suggest a bidirectional interaction exists between the estrogen/ER- and growth factor/MAPK-mediated signaling pathways in the regulation of proliferation even in cells in primary culture, and substantiate the findings of numerous other studies that have used estrogen-responsive cell lines. The mechanism by which MEK1/2 inhibitors inhibit estradiol-induced lactotroph proliferation may be via blockade of MAPK activation by growth factors that are released in a paracrine or autocrine manner in response to estradiol [19], involving the MAPK cascade to stimulate proliferation [48]. An alternative mechanism may involve blockade of MAPK activation by the direct action of estradiol on molecules upstream of MAPK, such as IGF-I receptors [49], Src [50], and Shc [51]. A possible mechanism to account for the inhibition of insulin-induced lactotroph proliferation by antiestrogens is by suppression of the ability of insulin/MAPK to activate ER-mediated transcription activity in a ligand-independent manner. It has been postulated that transcriptional activation by MAPK occurs through growth factor receptor-induced phosphorylation of the ER [32,52] or steroid receptor coactivators [53,54].

An interesting and unexpected finding in our study was that ICI182780 and OHT did not antagonize estradiol mitogenic action but instead enhanced this action when administered for the last 28 h of a 88 h estradiol treatment period. The finding that antiestrogens were effective in antagonizing estradiol mitogenic action when administered for the entire 88 h of estradiol treatment combined with the observation that the mitogenic action of estradiol does not occur with shorter times of treatment [26], suggest that the unexpected enhancement of estradiol action is dependent on the treatment times of both antiestrogens and estradiol. This enhancement occurs when short-term antiestrogen treatment is combined with long-term administration of estradiol. Several studies have shown that in addition to the mixed agonist/antagonist, even pure antiestrogen has agonistic activity on a variety of parameters, such as adenylate cyclase and MAPK activity and also on transcriptional activation at an activation protein-1 site when administered alone [35,36,55,56]. However, the proliferation-enhancing action of antiestrogen observed in the present study differed from these actions in that it occurred only in the presence of estradiol, thereby revealing an intriguing property of antiestrogens, the mechanism of which remains to be clarified. It is possible to account for this action on the basis of a novel interaction between the estrogen- and growth factor-mediated signaling pathways as indicated by the findings of our previous study on lactotrophs in primary culture [26]. This evidence includes the observations that estradiol had a paradoxical inhibitory effect on insulin- and IGF-I-induced proliferation with this antimitogenic action being antagonized by antiestrogens, and that the antimitogenic action was present after only 1 day of culture whereas 4 days were required for the well established mitogenic action of estradiol to occur. These findings indicate that the antimitogenic action of estradiol has a short latent period in comparison to long latencies for the mitogenic action of the hormone. When considering the mediation of the mitogenic action of long-term treatment with estradiol by paracrine or autocrine growth factors including IGF-I [19], it is possible that lactotroph proliferation at any given time reflects both the antimitogenic action with a short latency that occurs in the presence of growth factors, and the mitogenic action with a long latency. If this is the case, it would be anticipated that an enhanced proliferation in lactotrophs pre-treated with estradiol would be observed only after short-term antiestrogen treatment that acts to selectively block the antimitogenic action.

In conclusion, we have shown functional interactions in both directions between the cyclic AMP/PKA-, insulin/MAPK-, and estrogen/ER-mediated signaling pathways in the regulation of cell proliferation in estrogen-responsive cells grown in primary culture. These reciprocal cross-talks allow us to postulate the existence of a biochemical pathway shuttling between the ER and the PKA or MAPK pathways. Activation of this shuttling pathway would cause an amplification of signals generated initially upon estrogen binding to ERs, leading to marked stimulation of cell proliferation.

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