Endocrine, *vol. 13, no. 3, 385–392, December 2000* 0969–711X/00/13:385–392/$12.00

Involvement of Phosphoinositide-3-Kinase and p70 S6 Kinase in Regulation of Proliferation of Rat Lactotrophs in Culture

Kengo Kawashima, Koji Yamakawa, and Jun Arita

Department of Physiology, Yamanashi Medical University, Yamanashi, Japan

Phosphoinositide-3-kinase (PI-3K) and p70 S6 kinase ($p70^{S6k}$) are suggested as important molecules for mediating mitogenic actions of growth factors and cytokines in a variety of cell types. The purpose of the present study was to investigate whether these kinases were involved in mediation of the mitogenic actions of not only the growth factor insulin but also cyclic adenosine monophosphate (cAMP) and estrogen on rat cultured lactotrophs. Treatment with wortmannin or LY294002, a PI-3K inhibitor, or rapamycin, a $p70^{S6k}$ inhibitor, decreased basal levels of 5-bromo-2-deoxyuridine (BrdU)-labeling indices of lactotrophs in a dose-dependent manner. These inhibitors were effective in blocking an increase in BrdU-labeling indices induced by insulin. LY294002 and rapamycin also suppressed an increase in BrdU-labeling indices induced by forskolin, an adenylate cyclase activator, or dibutyryl cAMP, a membrane-permeable cAMP analog, as well as that induced by estradiol, a physiologic extracellular activator of lactotroph proliferation. However, the dibutyryl cAMP-, but not insulin-induced proliferation, acquired a resistance to LY294002 and rapamycin by pretreatment with bromocriptine, a dopaminergic agonist that is able to suppress lactotroph proliferation. These results suggest that the mitogenic actions of cAMP and estradiol on rat lactotrophs are mediated by PI-3K and $p70^{S6k}$, and that dopaminergic inhibition modifies the PI-3K and $p70^{S6k}$ dependence of the regulation of lactotroph proliferation.

Key Words: Phosphoinositide-3-kinase; p70 S6 kinase; lactotroph proliferation; cyclic adenosine monophosphate; estrogen; bromocriptine.

Received June 16, 2000; Revised July 31, 2000; Accepted August 7, 2000.
Author to whom all correspondence and reprints requests should be addressed: Dr. Jun Arita, Department of Physiology, Yamanashi Medical University, Tamaho, Yamanashi 409-3898, Japan. E-mail: jarita@res.yamanashi-med.ac.jp

Introduction

Proliferation of lactotrophs is regulated by extracellular factors derived from three distinct tiers. First, estradiol secreted from the ovaries stimulates lactotroph proliferation via direct and indirect actions (*1–5*). Second, hypothalamic hormones modify lactotroph proliferation by acting through hypophysial portal blood. The prolactin (PRL)-release inhibiting hormone dopamine (*6*), in particular, and its agonist, bromocriptine, are well known to inhibit lactotroph proliferation (*7–10*), and bromocriptine is widely used for medication of human PRL-secreting pituitary tumors (*11*). Third, anterior pituitary–derived growth factors act in an autocrine or paracrine manner on lactotrophs to change their proliferation (*12*). However, little is known about which intracellular signal transduction pathways mediate the mitogenic actions of these extracellular regulatory factors and how the distinct signal transduction pathways involved interact with each other. We have previously demonstrated that an increased concentration of intracellular cyclic adenosine monophosphate (cAMP) and the subsequent activation of protein kinase A (PKA) stimulate lactotroph proliferation, that bromocriptine inhibits lactotroph proliferation at least in part by suppressing the production of cAMP, and that the growth factor insulin stimulates lactotroph proliferation via activation of the mitogen-activated protein kinase (MAPK) cascade (*13*). Furthermore, interactions between the cAMP/PKA and MAPK pathways have been shown in relation to the regulation of lactotroph proliferation.

Phosphoinositide-3-kinase (PI-3K) is activated in response to growth factors and cytokines and regulates cell proliferation, cell survival, metabolic changes, and cytoskeletal organization by producing the second-messenger phosphatidylinositol-3,4,5-triphosphate (*14*). Inhibition of the PI-3K activity including treatment with PI-3K inhibitors such as wortmannin and LY294002 abolishes mitogen-induced proliferation in cell type– and mitogen-specific manners (*15–18*), suggesting that the kinase plays a regula-

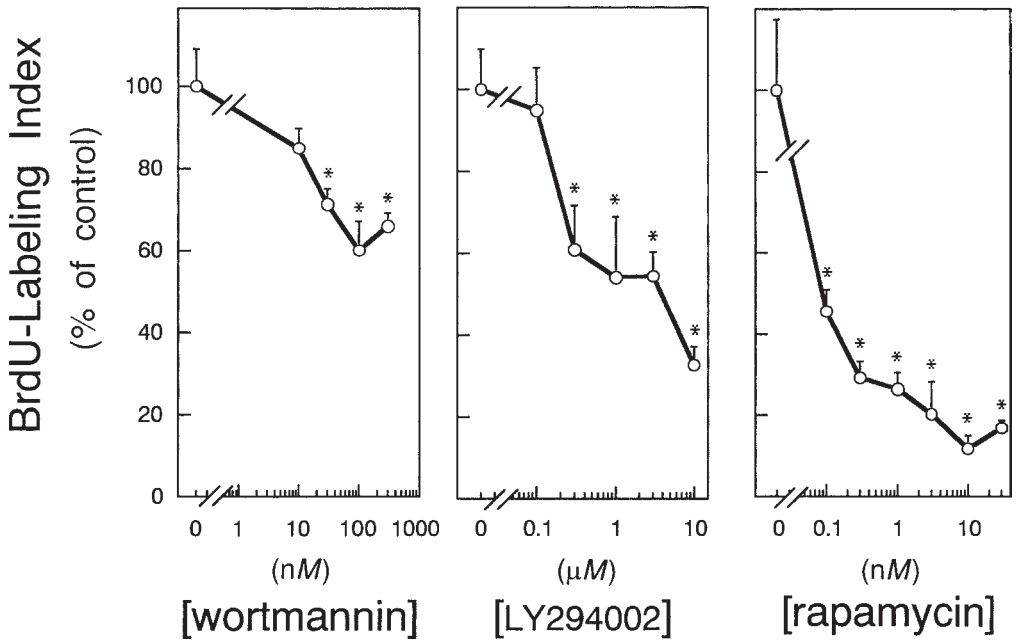

Fig. 1. Dose responses of lactotroph proliferation to the PI3K inhibitors wortmannin and LY294002 and the p70^{S6k} inhibitor rapamycin. Anterior pituitary cells that had been cultured in serum-free medium were treated with either vehicle or varying concentrations of wortmannin (*left*), LY294002 (*middle*), or rapamycin (*right*) for 42 h and were labeled with BrdU for 18 h before the end of treatment. BrdU-labeling indices of lactotrophs are expressed relative to vehicle-treated control groups. Data are the mean ± SEM of triplicate determinations from a representative experiment that was replicated three times with separate batches of cell preparation. *A significant difference at $p < 0.05$ compared with vehicle treated-groups.

tory role in proliferation. p70 S6 kinase (p70^{S6k}), first identified as a kinase that phosphorylates ribosomal protein S6, has been implicated in the regulation of not only protein translation but also gene expression and cell proliferation (*19*). Inhibition of the p70^{S6k} activity including treatment with rapamycin blocks cell proliferation induced by a variety of growth factors and cytokines (*20–22*). These results suggest that p70^{S6k} is an essential molecule for proliferation induced by virtually all mitogenic stimuli of growth factors, cytokines, and phorbol esters. Furthermore, mitogen-induced activation of p70^{S6k} has been shown to be mediated partly by its upstream kinase PI-3K (*23*).

In the present study, we investigated whether PI-3K and p70^{S6k} were involved in the regulation of proliferation of rat lactotrophs in culture, using inhibitors of these kinases. Although numerous studies have shown in detail the involvement of these kinases in the regulation of proliferation induced by growth factors and cytokines, little is known about their involvement in proliferation induced by cAMP and steroid hormones. In this regard, the lactotroph used in this study is a good experimental model because its proliferation has been shown to be stimulated by both cAMP and estradiol. To obtain the results under more physiologic conditions, we utilized cells in primary culture rather than tumor cell lines widely used in related studies because these tumor cells are not normal in the regulation of proliferation.

Results

Effects of Inhibitors of PI-3K and p70^{S6k} on Basal Lactotroph Proliferation

The dose responses of basal proliferation of lactotrophs to inhibitors of PI-3K and p70^{S6k} were determined. The mean 5-bromo-2-deoxyuridine (BrdU)-labeling index of lactotrophs under basal conditions in which cells were labeled with BrdU for 18 h was 2.6 ± 0.4% (mean ± SEM, based on six independent experiments). The basal levels of BrdU-labeling indices of lactotrophs were decreased after 42 h of treatment with wortmannin and LY294002, PI-3K inhibitors, in a dose-dependent manner. Wortmannin at 100–300 n*M* induced a maximal decrease of 40% in BrdU-labeling indices, compared with vehicle treatment (Fig. 1, left). LY294002 was more potent than wortmannin, revealing a maximal inhibition of 67% in BrdU-labeling indices at 10 μ*M* (Fig. 1 middle). The p70^{S6k} inhibitor rapamycin was highly effective in inhibiting basal proliferation of lactotrophs. BrdU-labeling indices were significantly decreased by rapamycin at as little as 0.1 n*M*, and a maximal decrease of 88% was obtained by 10 n*M* (Fig. 1, right). Based on these results, 100 n*M* wortmannin, 3 μ*M* LY294002, and 10 n*M* rapamycin were chosen for blocking the activities of PI-3K and p70^{S6k} in the subsequent experiments.

Effects of Inhibitors of PI-3K and p70^{S6k} on Insulin-Induced Lactotroph Proliferation

Wortmannin, LY294002, or rapamycin was added into medium 10 min before 42 h of treatment with 500 ng/mL of insulin. The inhibitory effects of pretreatment with 100 n*M* wortmannin, 3 μ*M* LY294002, and 10 n*M* rapamycin on basal proliferation were the same as those seen in Fig. 1. Treatment with insulin increased BrdU-labeling indices 2.6-fold above the basal level. The insulin-induced increase in BrdU-labeling indices was lowered to 56% by wortmannin, 31% by LY294002, and 11% by rapamycin ($p < 0.01$) (Fig. 2).

Effects of Inhibitors of PI-3K and p70^{S6k} on cAMP-Induced Lactotroph Proliferation

To determine whether PI-3K and p70^{S6k} were involved in lactotroph proliferation induced by increasing intracellular cAMP levels, the effects of LY294002 and rapamycin on BrdU-labeling indices of lactotrophs treated with dibutyryl cAMP (db-cAMP) and forskolin were examined. On the basis of our results of a previous study (*13*), doses of 1 m*M* for db-cAMP and 1 μ*M* for forskolin were chosen to effectively increase intracellular cAMP levels. Treatment with 1 m*M* db-cAMP and 1 μ*M* forskolin increased BrdU-labeling indices 3.3- and 3.8-fold, respectively, above the basal level. Pretreatment with 3 μ*M* LY294002 and 10 n*M* rapamycin decreased the db-cAMP-induced high levels of BrdU-labeling indices to 56 and 47%, respectively ($p < 0.01$) (Fig. 3). Likewise, the action of forskolin on BrdU-labeling indices was markedly inhibited to 39% by LY294002 and 15% by rapamycin ($p < 0.01$). We have previously shown that an increased intracellular cAMP level changes the cell shape of lactotrophs from round to flat polygonal cells (*13*). Because phase-contrast microscopy revealed that neither pretreatment with LY294002 nor rapamycin had effects on db-cAMP- or forskolin-induced changes in cell shape (data not shown), PI-3K and p70^{S6k} might not be required for the cAMP-induced change in cell shape.

Effects of Inhibitors of PI-3K and p70^{S6k} on Estradiol-Induced Lactotroph Proliferation

Treatment with estradiol at doses of 0.1–1.0 n*M* for 66 h was chosen to stimulate lactotroph proliferation on the basis of the results of preliminary experiments. Unlike the other experiments, lactotrophs were labeled with BrdU for 24 h in this experiment. Treatment for 66 h with 0.1 and 1.0 n*M* estradiol raised basal levels of BrdU-labeling indices of lactotrophs 4.7- and 6.1-fold, respectively, compared with vehicle treatment (Fig. 4). Both 3 μ*M* LY294002 and 10 n*M* rapamycin were highly effective in abolishing the increases in BrdU-labeling indices induced by 0.1 and 1.0 n*M* estradiol ($p < 0.01$).

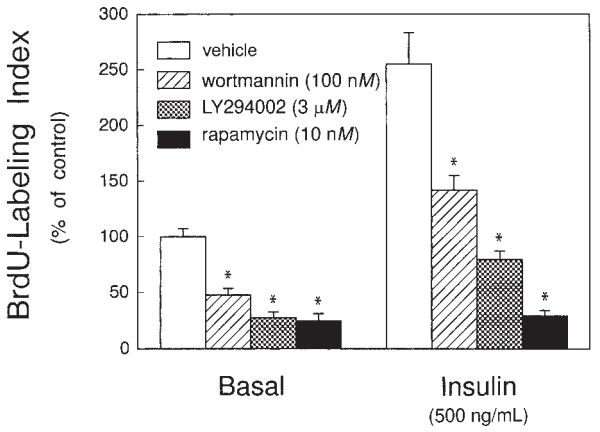

Fig. 2. Inhibition of insulin-induced lactotroph proliferation by wortmannin, LY294002, and rapamycin. Anterior pituitary cells that had been cultured in serum-free medium were pretreated with vehicle, 100 n*M* wortmannin, 3 μ*M* LY294002, or 10 n*M* rapamycin 10 min before treatment with either vehicle or 500 ng/mL of insulin for 42 h and were labeled with BrdU for 18 h before the end of treatment. BrdU-labeling indices of lactotrophs are expressed relative to a vehicle-treated control group under basal conditions. Data are the mean ± SEM of triplicate determinations from a representative experiment that was replicated three times with separate batches of cell preparation. *A significant difference at $p < 0.05$ compared with the corresponding vehicle-treated groups.

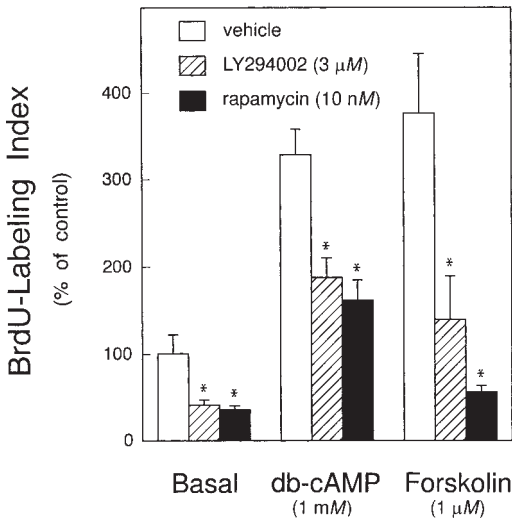

Fig. 3. Inhibition of cAMP-induced lactotroph proliferation by LY294002 and rapamycin. Anterior pituitary cells that had been cultured in serum-free medium were pretreated with vehicle, 3 μ*M* LY294002, or 10 n*M* rapamycin 10 min before treatment with vehicle, 1 m*M* db-cAMP, or 1 μ*M* forskolin for 42 h and were labeled with BrdU for 18 h before the end of treatment. BrdU-labeling indices of lactotrophs are expressed relative to a vehicle-treated control group under basal conditions. Data are the mean ± SEM of triplicate determinations from a representative experiment that was replicated three times with separate batches of cell preparation. *A significant difference at $p < 0.05$ compared with the corresponding vehicle treated-groups.

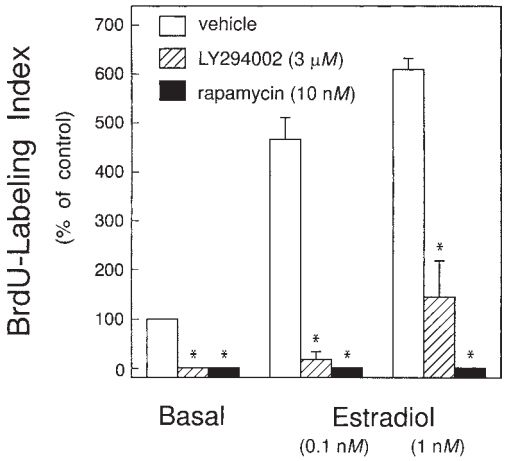

Fig. 4. Inhibition of estradiol-induced lactotroph proliferation by LY294002 and rapamycin. Anterior pituitary cells that had been cultured in serum-free medium were pretreated with vehicle, 3 μ*M* LY294002, or 10 n*M* rapamycin 10 min before treatment with vehicle or 0.1 or 1 n*M* estradiol for 66 h and were labeled with BrdU for 24 h before the end of treatment. BrdU-labeling indices of lactotrophs are expressed relative to a vehicle-treated control group under basal conditions. Data are the mean ± SEM of triplicate determinations from a representative experiment that was replicated three times with separate batches of cell preparation. *A significant difference at $p < 0.05$ compared with the corresponding vehicle-treated groups.

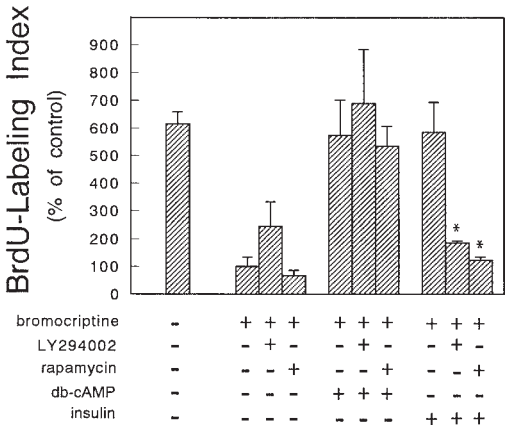

Fig. 5. Effects of bromocriptine pretreatment on the inhibitory actions of LY294002 and rapamycin on insulin- and cAMP-induced lactotroph proliferation. Anterior pituitary cells that had been cultured in serum-free medium were pretreated with or without 1 n*M* bromocriptine and 10 min later were treated with vehicle, 3 μ*M* LY294002, or 10 n*M* rapamycin followed by vehicle, 500 ng/mL of insulin, or 1 m*M* db-cAMP for 42 h. The cells were labeled with BrdU for 18 h before the end of treatment. BrdU-labeling indices of lactotrophs are expressed relative to a bromocriptine-pretreated, vehicle-treated control group under basal conditions. Data are the mean ± SEM of triplicate determinations from a representative experiment that was replicated three times with separate batches of cell preparation. *A significant difference at $p < 0.05$ compared with the group treated with bromocriptine and insulin.

Effects of Pretreatment with Bromocriptine on Actions of Inhibitors of PI-3K and p70^{S6k} on Lactotroph Proliferation

We examined whether pretreatment with bromocriptine modified the inhibitory actions of PI-3K and p070^{s6k} inhibitors on lactotroph proliferation. Pretreatment with 1 n*M* bromocriptine alone for 42 h clearly lowered BrdU-labeling indices of lactotrophs under basal conditions ($p < 0.01$). Pretreatment with bromocriptine also attenuated markedly increases in BrdU-labeling indices induced by 1 m*M* db-cAMP and 500 ng/mL of insulin, resulting in levels similar to basal ones in bromocriptine-untreated control lactotrophs. Treatment with 3 μ*M* LY294002 or 10 n*M* rapamycin did not decrease basal levels of BrdU-labeling indices of bromocriptine-pretreated lactotrophs (Fig. 5). Although 3 μ*M* LY294002 and 10 n*M* rapamycin were still effective in lowering the high levels of BrdU-labeling indices induced by insulin in the bromocriptine-pretreated lactotrophs ($p < 0.01$), the increase in BrdU-labeling indices induced by db-cAMP was changed by neither LY294002 nor rapamycin.

Discussion

To determine whether the actions of known mitogens on lactotrophs are mediated by PI-3K and p70^{S6k}, we used pharmacologic means. To suppress the activity of PI-3K, wortmannin, a fungal metabolite, and LY294002, a synthetic inhibitor, were used at concentrations of 100 n*M* and 3 μ*M*, respectively. Although they have proved valuable agents for studying PI-3K-dependent functions (*18,24*), there are several reports indicating that high doses of wortmannin suppress the activities of other enzymes such as myosin light chain kinase (*25*), phospholipase D (*26*), and double-stranded DNA-dependent protein kinase (*27*). Because the dose of wortmannin we used is lower than that reported to suppress the other enzymes, its actions on lactotroph proliferation seen in the present study seem specific for PI-3K. The specificity of wortmannin is also supported by the fact that the structurally unrelated PI-3K inhibitor LY294002 gave essentially the same results as wortmannin. Rapamycin is a highly specific inhibitor of p70^{S6k}, preventing phosphorylation and activation of p70^{S6k} by mitogens without effects on the closely related family member p90^{rsk} (*22*). Rapamycin does not suppress directly p70^{S6k} but target mTOR/FRAP (*28*), an immediately upstream molecule of this kinase. Because mTOR/FRAP also has been shown to activate 4E-BP1/PHAS, another regulatory molecule for mRNA translation (*19*), the possibility that the effects of rapamycin observed in the present study are attributed to inhibition of kinases other than p70^{S6k} that are involved in the regulation of proliferation cannot be excluded completely.

Wortmannin, LY294002, and rapamycin inhibited basal and insulin-induced proliferation of lactotrophs with doses comparable to those used in other studies (*15, 23, 29, 30*).

Potency of wortmannin less than that of LY294002 for inhibiting basal and insulin-induced lactotroph proliferation may be owing to the instability of wortmannin at the physiologic temperature of culture medium, as reported elsewhere (*31*). Insulin, a mitogen usually used in serum-free culture, is known to stimulate proliferation at high concentrations mostly through the type 1 insulin-like growth factor (IGF) receptor (*32*). Because there is evidence for the production of IGF-1 and the existence of the type 1 IGF receptor in the anterior pituitary gland (*33*) and stimulation of lactotroph proliferation by IGF-1 (*34*, unpublished data), IGF-1 is suggested to be an endogenous growth factor involved in the regulation of lactotroph proliferation. The results obtained with lactotrophs are in agreement with the established view that PI-3K and p70^{S6k} are involved in the regulation of proliferation induced by a variety of growth factors in a number of cell types (*14,19*).

cAMP inhibits proliferation in a variety of cell types but stimulates it in a limited number of cell types (*35*). Molecules involved in the signal transduction pathways of these cAMP actions are poorly understood except several molecules including p70^{S6k}, PI-3K, and MAPK. cAMP blocks interleukin-2 (IL-2) activation of p70^{S6k} and prevents proliferation in CTLL-20 lymphoid cells (*30*), whereas p70^{S6k} activation and mitogenesis are induced by cAMP in WRT thyroid cells and Swiss 3T3 fibroblasts (*29,36*). These results indicate a correlation between the activity of p70^{S6k} and the antimitogenic or mitogenic action of cAMP. Furthermore, rapamycin has been shown to abolish cAMP-induced p70^{S6k} activation and proliferation in the Swiss 3T3 cell line (*29,36*). The effectiveness of rapamycin on cAMP-induced cell proliferation was confirmed by the present study using lactotrophs in primary culture. Based on these findings, p70^{S6k} appears to be required for mediation of not only the mitogenic actions of growth factors and cytokines but also that of cAMP. A few reports suggest involvement of PI-3K in the cAMP actions. In the CTLL-20 cells, IL-2-induced activation of PI-3K is suppressed by cAMP (*30*), although PI-3K activation by cAMP has not yet been demonstrated in cells in which cAMP stimulates proliferation. The present study demonstrates that LY294002 blocks cAMP-induced lactotroph proliferation in primary culture. In the WRT cell line, PI-3K inhibitors block both cAMP-induced p70^{S6k} activation and proliferation (*29*), suggesting that PI-3K is an upstream regulator of p70^{S6k} regarding the mitogenic action of cAMP as well as growth factors and cytokines.

It is controversial whether the MAPK cascade is involved in mediation of the cAMP actions on proliferation. cAMP has been shown to suppress growth factor-induced MAPK activation and proliferation in several cells (*37,38*). However, whether the MAPK is required for the cAMP-induced p70^{S6k} activation and proliferation appears cell type dependent. The actions of cAMP on proliferation and p70^{S6k} activation are not mediated by the MAPK cascade in the WRT and Swiss 3T3 cells (*36,39*), and our previous study showed that the MEK inhibitor PD98059 inhibited lactotroph proliferation induced by not only insulin but also cAMP (*13*). Based on our results of the previous and present studies, it is possible that in lactotrophs, the MAPK cascade lies at a point of convergence of the PI-3K/p70^{S6k} and cAMP/PKA pathways in the regulation of proliferation. Indeed, recent findings have provided evidence suggesting the existence of a pathway that consists of PI-3K, MAPK, and p70^{S6k} (*40–42*).

The present study demonstrates that estradiol-induced lactotroph proliferation is blocked by LY294002 and rapamycin. To our knowledge, this result is the first to suggest involvement of PI-3K and p70^{S6k} in steroid hormone–stimulated cell proliferation. Although the signal transduction pathway through which estradiol stimulates cell proliferation is not fully understood, studies on uterine and mammary cells have shown that growth stimulation by estradiol is an indirect process mediated by estradiol-induced, autocrine-acting growth factors, such as epidermal growth factor, IGF1, and transforming growth factor (*43*). Considering such an indirect regulation by estradiol of cell proliferation, the inhibition by LY294002 and rapamycin of estradiol-induced lactotroph proliferation observed in the present study may merely reflect the effects of these inhibitors on PI-3K and p70^{S6k} activation by an as-yet unidentified growth factor secreted in response to estradiol. Alternatively, numerous studies have suggested that estradiol rapidly stimulates expression of protooncogenes such as c-*myc*, c-*fos*, and c-*jun* that are able to induce proliferation (*44–46*). Therefore, it is possible that PI-3K and p70^{S6k} are involved, at some point, in a protooncogene-mediated pathway of the mitogenic action of estradiol. Whatever mechanism the mitogenic action of estradiol involves in lactotrophs, it remains to be determined whether estradiol induces phosphorylation and activation of p70^{S6k} and whether PI-3K mediates the estradiol action.

Clear differences in the effectiveness of each inhibitor are indicated by the results that rapamycin fully inhibited lactotroph proliferation induced by insulin, forskolin, and estradiol whereas only partial inhibition was observed by LY294002. The difference between the effectiveness of rapamycin and LY294002 observed in the present study might be accounted for by the existence of some other signal transduction pathways in parallel with the PI-3K pathway at the upstream of p70^{S6k}. Indeed, the mitogenic action of platelet-derived growth factor has been shown to be mediated independently by PI-3K and phospholipase C pathways, both of which converge upon the downstream effector p70^{S6k} (*17,23*).

Interestingly, in the presence of bromocriptine, an antimitogenic agent of lactotrophs, LY294002, and rapamycin had differential effects on cAMP- and insulin-induced

lactotroph-proliferation: these inhibitors failed to block the db-cAMP, but not the insulin-induced lactotroph proliferation. This result is unexpected because rapamycin abolishes the actions of virtually all mitogens except that of IGF-2 on L6E9 myoblasts (*47*), and no agent is known to antagonize an antimitogenic effect of rapamycin. Our result suggests that in the presence of dopaminergic inhibition, PI-3K and p70^{S6k} are not involved in the regulation of the cAMP-induced proliferation and that, unlike insulin, cAMP is able to stimulate lactotroph proliferation via a PI-3K/p70^{S6k}-independent signal transduction pathway. The presence of the PI-3K/p70 ^{S6k}-independent signal transduction pathway is also suggested by the result that the inhibitors used were not able to block completely lactotroph proliferation induced by the various mitogens. Indeed, the partial inhibition by LY294002 might be accounted for by phospholipase C, an upstream regulator of p70^{S6k}, which has been shown to be involved in the mitogenic regulation in parallel to PI-3K (*19*). Because heterogeneity of lactotrophs has been well documented (*48*), it is possible that such an alternative pathway is utilized by a different lactotroph subpopulation as exemplified in somatotrophs (*49*).

In conclusion, the activities of PI-3K and p70^{S6k} are required for lactotroph proliferation induced by not only growth factors including insulin but also cAMP and estradiol. However, under conditions in which bromocriptine inhibits lactotroph proliferation, the regulation of lactotroph proliferation seems less dependent on PI-3K and p70^{S6k}.

Materials and Methods

Cell Culture

Experiments were conducted under the guidelines of the Ethical Committee of Animal Experiments in Yamanashi Medical University. Six-week-old female Wistar rats purchased from Japan SLC (Shizuoka, Japan) were used for primary cell cultures. Anterior pituitary cells were dispersed as described previously (*10*). Briefly, five anterior pituitaries were minced for suspension in minimal essential medium (MEM) (Sigma, St. Louis, MO) containing $NaHCO_3$, penicillin G, streptomycin, bovine serum albumin (BSA), and HEPES. They were incubated at 37°C in the MEM containing 0.01% trypsin (TRL-3; Worthington, Freehold, NJ) and 0.005% DNase in a siliconized Spinner suspension flask with constant stirring for 90 min. The dispersed pituitary cells were treated with trypsin inhibitor and DNase and subjected to cell counting and viability test. A 100-μL aliquot of cell suspension containing 1.5×10^5 cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20 m*M* HEPES was placed on poly-D-lysine-coated 35-mm culture dishes (Falcon®; Becton Dickinson, Bedford, MA). The cells were subsequently allowed to attach to the surface of the dishes in a humidified CO_2 incubator for 1 h. The pituitary cells were flooded with 2 mL of DMEM containing HEPES, 8.3% horse serum, 1.7% fetal bovine serum, penicillin, and streptomycin and precultured at 37°C in a humidified atmosphere of 5% CO_2 and 95% air for 1 d. After the preculture with medium containing serum, the pituitary cells were washed with a 1:1 mixture of DMEM and Ham's Nutrient Mix F-12 without phenol red and containing 15 m*M* HEPES, penicillin, and streptomycin (DMEM/F12), and serum-free cultures were initiated with a serum-free, chemically defined medium (the initiation day was designated as d 1 of the experiment). The serum-free medium was originally derived from medium described elsewhere (*10,50*), with some modifications: DMEM/F12 supplemented with 10 μg/mL of bovine transferrin, 40 n*M* sodium selenite, 30 μ*M* ethanolamine, 100 p*M* triiodothyronine, 5 m*M* ethanol, and 0.1% BSA. The serum-free medium was freshly changed on d 2 and 4 during the culture period.

Treatment of Lactotrophs in Culture

Forskolin (Sigma) and bromocriptine methylate (Sigma) were initially dissolved with ethanol at concentrations of 20 and 1 m*M*, respectively. db-cAMP was dissolved with Milli Q water at a concentration of 100 m*M*. Bovine insulin (Sigma) was dissolved with the medium. Wortmannin (RBI, Natick, MA), LY294002 (Sigma), and rapamycin (Sigma) were dissolved with dimethylsulfoxide (DMSO) at concentrations of 2, 100, and 1 m*M*, respectively. These agents were diluted immediately before use, the final concentrations of DMSO in medium being 0.1–0.25%. Cultured cells received 42-h treatments with the inhibitors and mitogens on d 2 and 18-h treatment with 200 μ*M* BrdU for labeling proliferating cells on d 3. However, in experiments in which the effects of the inhibitors on estradiol-induced lactotroph proliferation were examined, the culture conditions were changed in part to maximize the mitogenic action of estradiol; pituitary cells were precultured with DMEM supplemented with 500 ng/mL of insulin instead of serum, and the treatment time with estradiol was prolonged to 66 h, resulting in BrdU treatment on d 4 and completion of culture on d 5.

At the end of culture on d 4 (d 5 in estradiol experiments), cultured pituitary cells were detached from culture dishes and redispersed with trypsin as described previously (*10*). Cells suspended with Earle's balanced salt solution containing HEPES were attached to poly-D-lysine-coated glass slides by centrifugation with a cytocentrifuge (SC-2; Tomy, Tokyo, Japan). The cells attached on the glass slides were fixed with ice-methanol and stored in phosphate-buffered saline (PBS) at 4°C until immunostaining.

Immunostaining

The anterior pituitary cells attached to glass slides were double immunostained for BrdU and PRL, as described previously (*51*). Briefly, the slides were treated with 3 *M* HCl for 30 min and 10% normal donkey serum in PBS for 20 min. Double labeling immunofluorescence staining was

performed by three steps using the following reagents: (1) a mixture of mouse monoclonal anti-BrdU (Sigma) at 1:200 dilution and rabbit anti-rat PRL (NIDDK IC-5) at 1:4000 dilution; (2) horse biotinylated anti-mouse IgG (Vector, Burlingame, CA) at 1:50 dilution; and (3) a mixture of Texas Red–labeled streptavidin (Amersham, Arlington Heights, IL) at 1:50 dilution and fluorescein isothiocyanate (FITC)-labeled donkey anti-rabbit IgG (Amersham) at 1:50 dilution. The cells were incubated with 100 μL of each reagent diluted with PBS containing 10% normal donkey serum for 1 h followed by 20 min of washing.

The immunostained slides were observed with a fluorescence microscope (BX50-FLA; Olympus, Tokyo, Japan) equipped with a dual band mirror unit for FITC and Texas Red (U-DM-FI/TX).

Statistical Analyses

A total of 1000–2000 PRL-immunoreactive cells were examined in randomly chosen fields for each slide to determine the BrdU-labeling index, which was the percentage of pituitary cells immunoreactive for both PRL and BrdU of total PRL-immunoreactive cells counted. Three slides were analyzed for each treatment group derived from the same cell preparations. Experiments were replicated three to four times with separate batches of cell preparations. Differences between groups were statistically analyzed using one-way analsysis of variance followed by Fisher's Protected Least Significant Difference test.

Acknowledgments

We thank Dr. A. F. Parlow and the NIDDK for providing prolactin antiserum for immunocytochemistry and W. Takahashi for her expert technical assistance. This work was supported by the Ministry of Education, Science and Culture of Japan (Grant-in-Aid for Scientific Research 10670060, 12770907).

References

1. Wiklund, J., Wertz, N., and Gorski, J. (1981). *Endocrinology* **109**, 1700–1707.
2. Lieberman, M. E., Maurer, R. A., Claude, P., and Gorski, J. (1982). *Mol. Cell. Endocrinol.* **25**, 277–294.
3. Sarkar, D. K., Gottschall, P. E., and Meites, J. (1982). *Science* **218**, 684–686.
4. Song, J., Jin, L., and Lloyd, R. V. (1989). *Cancer Res.* **49**, 1247–1253.
5. Arita, J., Kojima, Y., and Kimura, F. (1990). *Neuroendocrinology* **52**, 249–255.
6. Ben-Jonathan, N. (1985). *Endocr. Rev.* **6**, 564–589.
7. MacLeod, R. M. and Lehmeyer, J. E. (1973). *Cancer Res.* **33**, 849–855.
8. Lloyd, H. M., Meares, J. D., and Jacobi, J. (1975). *Nature* **255**, 497–498.
9. Melmed, S. (1981). *Endocrinology* **109**, 2258–2260.
10. Arita, J., Hashi, A., Hoshi, K., Mazawa, S., and Suzuki, S. (1998). *Neuroendocrinology* **68**,163–171.
11. Bevan, J. S., Webster, J., Burke, C. W., and Scanlon, M. F. (1992). *Endocr. Rev.* **13**, 220–240.
12. Ray, D., and Melmed, S. (1997). *Endocr. Rev.* **18**, 206–228.
13. Suzuki, S., Yamamoto, I., and Arita, J. (1999). *Endocrinology* **140**, 2850–2858.
14. Carpenter, C. L. and Cantley, L. C. (1996). *Biochim. Biophys. Acta.* **1288**, M11–M16.
15. Karnitz, L. M., Burns, L. A., Sutor, S. L., Blenis, J., and Abraham, R. T. (1995). *Mol. Cell. Biol.* **15**, 3049–3057.
16. Roche, S., Koegl, M., and Courtneidge, S. A. (1994). *Proc. Natl. Acad. Sci. USA* **91**, 9185–9189.
17. Valius, M. and Kazlauskas, A. (1993). *Cell* **73**, 321–334.
18. Vlahos, C. J., Matter, W. F., Hui, K. Y., and Brown, R. F. (1994). *J. Biol. Chem.* **269**, 5241–5248.
19. Chou, M. M. and Blenis, J. (1995). *Curr. Opin. Cell Biol.* **7**, 806–814.
20. Kuo, C. J., Chung, J., Fiorentino, D. F., Flanagan, W. M., Blenis, J., and Crabtree, G. R. (1992). *Nature* **358**, 70–73.
21. Lane, H. A., Fernandez, A., Lamb, N. J. C., and Thomas, G. (1993). *Nature* **363**, 170–172.
22. Price, D. J., Grove, J. R., Calvo, V., Avruch, J., and Biere, B. E. (1992). *Science* **257**, 973–977.
23. Chung, J., Grammer, T. C., Lemon, K. P., Kazlauskas, A., and Blenis, J. (1994). *Nature* **370**, 71–75.
24. Ui, M., Okada, T., Hazeki, K., and Hazeki, O. (1995). *Trends Biochem. Sci.* **20**, 303–307.
25. Yano, H., Nakanishi, S., Kimura, K., Hanai, N., Saitoh, Y., Fukui, Y., Nonomura, Y., and Matsuda, Y. (1993). *J. Biol. Chem.* **268**, 25,846–25,856.
26. Bonser, R. W., Thompson, N. T., Randall, R. W., Tateson, J. E., Spacey, G. D., Hodson, H. F., and Garland, L. G. (1991). *Br. J. Pharmacol.* **103**, 1237–1241.
27. Hunter, T. (1995). *Cell* **83**, 1–4.
28. Brown, E. J., Beal, P. A., Keith, C. T., Chen, J., Shin, T. B., and Schreiber, S. L. (1995). *Nature* **377**, 441–446.
29. Cass, L. A. and Meinkoth, J. L. (1998). *Endocrinology* **139**, 1991–1998.
30. Monfar, M., Lemon, K. P., Grammer, T. C., Cheatham, L., Chung, J., Vlahos, C. J., and Blenis, J. (1995). *Mol. Cell. Biol.* **15**, 326–337.
31. Kimura, K., Hattori, S., Kabuyama, Y., Shizawa, Y., Takayanagi, J., Nakamura, S., Toki, S., Matsuda, Y., Onodera, K., and Fukui, Y. (1994). *J. Biol. Chem.* **269**, 18,961–18,967.
32. Sara, V. R. and Hall, K. (1990). *Physiol. Rev.* **70**, 591–614.
33. Bach, M. A. and Bondy, C. A. (1992). *Endocrinology* **131**, 2588–2594.
34. Geary, E. S., Lim, M., Ceda, G. P., Ro, S., Rosenfeld, R. G., and Hoffman, A. R. (1989). *J. Neuroendocrinol.* **1**, 179–184.
35. Roger, P. P., Reuse, S., Maenhaut, C., and Dumont, J. E. (1995). *Vitam. Horm.* **51**, 59–191.
36. Withers, D. J., Bloom, S. R., and Rozengurt, E. (1995). *J. Biol. Chem.* **270**, 21,411–21,419.
37. Cook, S. J. and McComick, F. (1993). *Science* **262**, 1069–1072.
38. Wu, J., Dent, P., Jelinek, T., Wolfman, A., Weber, M. J., and Sturgill, T. W. (1993). *Science* **262**, 1065–1069.
39. Miller, M. J., Prigent, S., Kupperman, E., Rioux, L., Park, S.-H., Feramisco, J. R., White, M. A., Rutkowski, J. L., and Meinkoth, J. L. (1997). *J. Biol. Chem.* **272**, 5600–5605.
40. Duckworth, B. C. and Cantley, L. C. (1997). *J. Biol. Chem.* **272**, 27,665–27,670.
41. Lopez-llasaca, M., Crespo, P., Pellici, P. G., Gutkind, J. S., and Wetzker, R. (1997). *Science* **275**, 394–397.
42. Mukhopadhyay, N. K., Price, D. J., Kyriakis, J. M., Pelech, S., Sanghera, J., and Avruch, J. (1992). *J. Biol. Chem.* **267**, 3325–3335.
43. Dickson, R. B. and Lippman, M. E. (1995). *Endocr. Rev.* **16**, 559–589.
44. Watson, P. H., Pon, R. T., and Shiu, R. P. C. (1991). *Cancer Res.* **51**, 3996–4000.

45. Webb, D. K., Moulton, B. C., and Khan, S. A. (1990). *Biochem. Biophys. Res. Commun.* **168**, 721–726.
46. Weisz, A. and Bresciani, F. (1988). *Mol. Endocrinol.* **2**, 816–824.
47. Canicio, J., Gallardo, E., Illa, I., Testar, X., Palacín, M., Zorzano, A., and Kaliman, P. (1998). *Endocrinology* **139**, 5042–5049.
48. Neill, J. D., Smith, P. F., Luque, E. H., de Toro, M. M., Nagy, G., and Mulchahey, J. J. (1987). *Recent Prog. Horm. Res.* **43**, 175–229.
49. Ramírez, J. L., Castaño, J. P., Torronteras, R., Martínez-Fuentes, A. J., Frawley, L. S., Gacía-Navarro, S., and Gracia-Navarro, F. (1999). *Endocrinology* **140**, 1752–1759.
50. Denef, C., Baes, M., and Schramme, C. (1984). *Endocrinology* **114**, 1371–1378.
51. Hashi, A., Mazawa, S., Kato, J., and Arita, J. (1995). *Endocrinology* **136**, 4665–4671.