Okadaic Acid Mimics Several Proximal Effects of Prolactin in Nb2 Lymphoma Cells (44316)

G. B. Carey[1] and J. P. Liberti[2]

Department of Biochemistry and Molecular Biophysics, Medical College of Virginia Campus, Virginia Commonwealth University, Richmond, Virginia 23298-0614

Abstract. **We previously reported that prolactin-mediated macromolecular synthesis and mitogenesis are coupled to the activation of mitogen-activated protein kinase (MAPK) and p70 S6-kinase ($p70^{S6K}$). Full activation of MAPK requires tyrosine and threonine phosphorylation whereas that of $p70^{S6K}$ requires serine phosphorylation. In the present study, okadaic acid, which inhibits serine/threonine protein phosphatase activity, was used to explore the linkage of MAPK and $p70^{S6K}$ activation to downstream effects of prolactin in Nb2 cells. The results show that 1 n*M* okadaic acid augmented prolactin-stimulated mitogenesis and synthesis of protein and DNA 250%, 42%, and 70%, respectively. Addition of okadaic acid alone a) stimulated and sustained $p70^{S6K}$ activity (5- to 8-fold) and MAPK (3.5- to 5-fold); and b) increased protein synthesis with the maximum effect being about equal to that of prolactin (2.1-fold with 1 n*M* okadaic acid versus 2.3-fold with 0.2 n*M* prolactin). However, okadaic acid did not affect DNA synthesis or mitogenesis. These results indicate that the activation of MAPK and $p70^{S6K}$ is necessary for stimulation of protein synthesis but not sufficient for prolactin-driven mitogenesis.** [P.S.E.B.M. 1998, Vol 219]

Reversible phosphorylation of proteins is an essential element in the regulation of both normal and neoplastic cell growth. The phosphorylation state of a protein depends upon the relative activities of protein kinase and phosphoprotein phosphatase (PPase). Considerable effort has focused on the kinases whereas the PPases have been relatively neglected. Although it is generally thought that PPases are constitutively active enzymes that reverse the effects of mitogen-stimulated kinases, recent results suggest a more active role of PPases in mitogen signaling (1–3). For example, tyrosine phosphorylation of PP2A, an essential enzyme distributed among all eukaryotes, occurs in intact cells and increases in response to serum or EGF stimulation (1). Protein phosphatase 2A also plays a role in the regulation of many cellular proteins, kinases, and hormone receptors as well as in growth (3) and cell division (1). A number of protein kinases that participate in mitogen signaling (e.g., $p70^{S6K}$), MAPKs (p42, p44, p54) and other insulin-stimulated kinases are inactivated by PP2A (4).

Prolactin signaling involves JAK2, a member of the Janus family of TKs (5). Subsequent to JAK2 activation, cytoplasmic TK(s), Raf-1, MAPK, and $p70^{S6K}$ are transiently activated (6, 7). MAPK requires phosphorylation of both tyrosine and threonine residues for maximum activity whereas $p70^{S6K}$ requires only serine phosphorylation. Of the several classes of ser/thr PPases, Types 2A and 1 are perhaps the best characterized (8). Okadaic acid preferentially inhibits type 2A PPase.

Using agents that selectively inhibit JAK2, MAPK, and $p70^{S6K}$, we reported that Prl-mediated macromolecular synthesis and mitogenesis were tightly coupled to JAK2 and MAPK activation whereas the linkage between $p70^{S6K}$ and mitogenesis was less stringent (9). For example, inhibition of JAK2 and MAPK abrogated Prl-induced anabolism and mitogenesis. On the other hand, complete inhibition of $p70^{S6K}$ attenuated protein synthesis and only delayed mitogenesis.

Based on the above, the effect of PP2A inhibition on the coupling of Prl-generated proximal signals to the distal

[1] Present address: The Guthrie Research Foundation, Sayre, PA 18840.
[2] To whom requests for reprints should be addressed at Department of Biochemistry and Molecular Biophysics, Medical College of Virginia Campus, Virginia Commonwealth Univ., Richmond, VA 23298-0614. E-mail: jliberti@hsc.vcu.edu

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effects of the lactogen was examined. The major findings showed that OA alone mimics several effects of Prl in Nb2 cells (i.e., stimulation of protein synthesis and activation of MAPK and $p70^{S6K}$) but does not affect DNA synthesis or mitogenesis. Okadaic acid markedly potentiates the effect of Prl on MAPK and $p70^{S6K}$ activation and mitogenesis. Stimulation of MAPK and $p70^{S6K}$ (ca. 2.5- to 3-fold), which is transient with Prl, is greatly enhanced (ca. 6- to 9-fold) and sustained when cells were exposed to Prl and OA. These results indicate that activation of MAPK and $p70^{S6K}$ is required for the stimulation of protein synthesis but is not sufficient for mitogenesis and suggest that additional signals, which are insensitive to PP2A, are necessary for Prl-mediated mitogenesis.

Materials and Methods

Materials. Ovine Prl (NIAMDD-15) was obtained from the National Institute of Diabetes, Digestive and Kidney Diseases (NIH). RPMI 1640 tissue culture medium, protease inhibitors, MBP, PKI, and calmidazolium were from Sigma Chemical Co., (St. Louis, MO). Fetal bovine and horse sera, the latter screened for the absence of lactogenic activity, were obtained from Whittaker M.A. Bioproducts (Walkersville, MD). Okadaic acid was purchased from LC Laboratories (Woburn, MA). Antibiotics and a PPase assay kit were obtained from GIBCO (Grand Island, NY). [^{35}S]methionine (1175Ci/mmol), [γ-^{32}P]ATP (3000Ci/mmol) and [^{3}H]thymidine (6.7Ci/mmol), were from NEN (Boston, MA). An S6 peptide (RRRLASLRA) corresponding to S6 residues 231–239, Ala^{235} (11) was synthesized by our department's Protein/Peptide Core Facility.

Nb2 Lymphoma Cell Culture. Lactogen-dependent Nb2 cells were maintained as described (10) except that RPMI 1640 was used in place of Fischer's medium (9). Prior to each experiment, cultures were growth-arrested by incubating cells for 22hr in medium used for stock cultures but without fetal bovine serum. Under these conditions, ca. 85% of the cells are arrested in G_o/G_I (12).

Nb2 Mitogenesis. Nb2 cells at 0.25 × 10^6/ml (in 1 ml; Falcon 24-well plates, or Costar 48-well plates) were incubated with vehicle (control) or combinations of Prl (1–1000 p*M*) and OA (0.1–10 n*M*). Aliquots of suspensions were removed at 48 and/or 72hr and cell number and viability were measured by trypan blue exclusion. Cell viability, determined at the end of all incubations, showed that OA and the diluent were not cytotoxic.

Measurement of DNA Synthesis. Cells (0.5 × 10^6/ml) were incubated with combinations of Prl and OA for 8hr. [^{3}H]thymidine (1 μCi/ml) was added, and the incubation was terminated at 16hr. Incorporation of radioactivity into acid-insoluble material was determined as described (9). Briefly, aliquots of incubates were washed with cold medium prior to the addition of 10% TCA containing 1 m*M* unlabeled thymidine. Pellets were washed with 5% TCA/unlabeled thymidine and then digested with formic acid. The entire tube contents were transferred to scintillation vials, and the radioactivity was determined.

Measurement of Protein Synthesis. Cell suspensions (0.5 × 10^6/ml) were incubated with combinations of Prl and OA in the presence of 2 μCi/ml [^{35}S]-methionine. The incorporation of radioactivity into TCA-insoluble material was determined by liquid scintillation counting as described (9).

Preparation of Cellular Extracts for Kinase Assays. Cell extracts were prepared as described (9). Briefly, cells were pelleted and resuspended in lysis buffer, pH 7.3, containing kinase and protease inhibitors. Lysates were prepared by sonication of the cell suspension using a Heat Systems Ultrasonics Inc. Sonicator (Farmingdale, NY) with three cycles each of 2-s bursts at 60% duty at a power setting of 4. Cytosol fractions were prepared by centrifuging the sonicates at 100,000*g* for 1 hr. Protein concentration of cytosol extracts was measured by the Bradford reagent using bovine serum albumin as standard.

***In vitro* Kinase Assays.** Enzyme reactions were conducted as described (9). Briefly, about 2 × 10^6 cell equivalents were assayed per reaction volume (75 μl). Phosphorylation was determined at ambient temperature in the presence of various protein kinase and protein phosphatase inhibitors. Substrates included MBP (1 mg/ml) and S6 peptide (250 μ*M*) (9, 13). Control reactions did not contain substrate. Reactions were initiated by the addition of 12 μCi [γ-^{32}P]ATP (sp act 1600 mCi/mmol) and were terminated in the linear portion of the curve (determined in pilot studies) by the addition of 15 μl of 33% TCA. The extent of phosphorylation (i.e., radioactivity bound to P-81 filter paper) was determined as described (9). Briefly, 75 μl aliquots of the TCA supernate were applied to the filter and washed with a solution containing 1 m*M* $Na_4P_2O_7$, 1 m*M* unlabeled ATP, 100 m*M* H_3PO_4, and 5% TCA. Papers were then counted in a scintillation counter.

Preparation of Cellular Extracts for Phosphatase Assays. Growth-arrested cells were concentrated to 2.5 × 10^6/ml. After re-suspension in 6-well, tissue culture plates, the cell suspensions (10 ml) were equilibrated for 30 min at 37°C. Incubations were done in the presence and absence of Prl with and without OA. When appropriate, cells were treated with OA for 15 min prior to the addition of mitogen. Cells were harvested on ice and lysed in PPase extraction buffer (kinase buffer without α-glycerophosphate and vanadate) essentially as described for the kinase assays (9, 13). Cytosol extracts were prepared as described above.

Measurement of Protein Phosphatase Activity. Assays were conducted using the *Protein Phosphatase Assay System* (Cat.# 13188-016, Gibco/BRL), as per the supplier's instructions. In this assay the enzymatic hydrolysis of ^{32}P-phosphate from the substrate, ^{32}P-labeled phosphorylase a, is quantified by determining the radioactivity of the released ^{32}P-inorganic phosphate. Reaction mixtures contained 10 μg of cell extract protein (20 μl: about 0.7 × 10^6 cell equivalents/ml), 20 μl substrate (^{32}P-phosphorylase-a)

and 40 μl PPase assay buffer containing protease inhibitors (9, 13). Reactions were initiated by the addition of substrate and conducted at 30°C. Inhibitors were diluted in PPase buffer and pre-incubated for 5 min at 30°C with reaction mixture minus substrate. Reactions were terminated at 10 min with 10% ice-cold TCA containing 1 mM H_3PO_4. BSA (final concentration 1 mg/ml) was then added. Precipitates were mixed, incubated at 4°C for 30 min, and centrifuged at 12,000g for 5 min. The radioactivity in the supernate, which contains ^{32}P-inorganic phosphate, was determined by liquid scintillation counting.

Data are expressed as the mean ± SE or as the ratio of experimental to control. The comparison between groups was analyzed by Student's t test with $P < 0.05$ taken as significant.

Results

The results (Fig. 1) show that growth-arrested Nb2 cells constitutively express relatively high levels of PPase activity (2400 pmol phosphate released/μg protein/min). Treatment with 1 nM Prl for 4hr, conditions which lead to the activation of several signaling events (9, 14), does not alter PPase activity. This lack of effect may be due to the use of unfractionated lysates and the assay that detects all divalent cation-independent ser/thr protein phosphatases. Addition of 100 nM OA inhibits PPase activity of basal and Prl-treated cells similarly with maximum inhibition (ca. 70%) obtained at 4hr. The effect of OA is concentration-dependent with 50% inhibition occurring at 1 nM (data not shown). As seen in Fig. 1, there is significant residual PPase activity at 4 hr. Exposure of these extracts to exogenously added OA (100 nM) inhibits PPase activity virtually 100% (data not shown). These results suggest that the majority of the phosphoesterase activity measured in Nb2 cells is ser/thr activity.

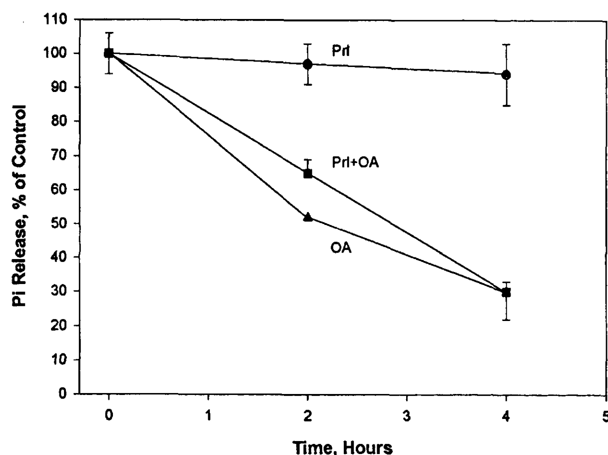
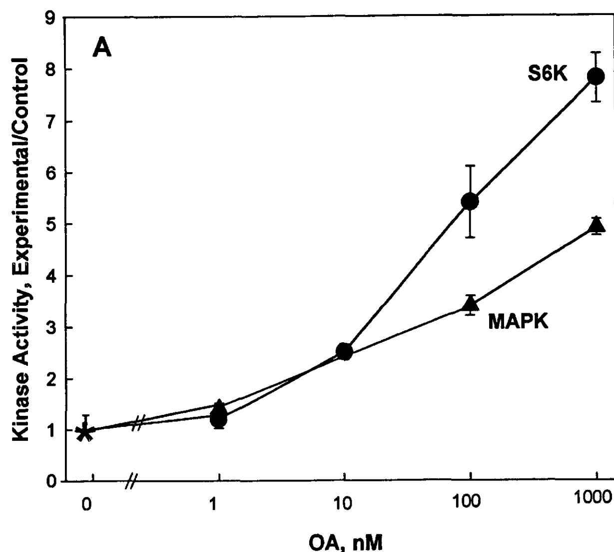


Figure 1. Effect of okadaic acid on protein phosphatase activity in Nb2 cells. Nb2 cells (10ml suspension at 2.5 x 10^6/ml) were treated with or without prolactin (Prl, 1 nM) in the presence or absence of okadaic for various times. Protein phosphatase activity at Time 0 was 2.4 ± 0.15 pmol phosphate released/μg protein/min. Results are expressed as percentage of control and are representative of at least four separate, independent experiments each for Prl or OA individually and two each for Prl with OA. The inhibitory effect of OA was significant at 2 and 4hr, $P < 0.02$.



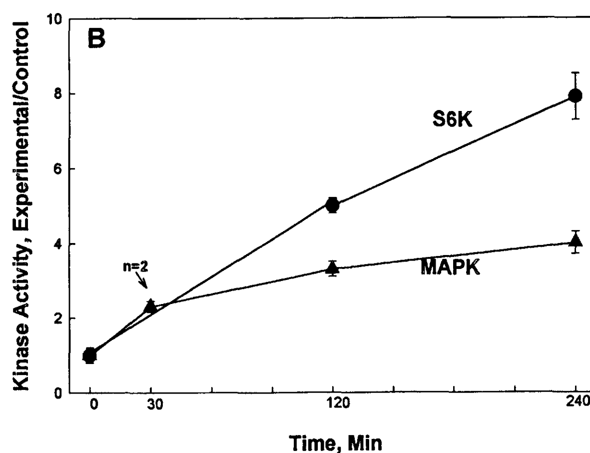


Figure 2. Effect of okadaic acid on p70^{S6K} and MAP kinase activities in Nb2 cells. (A) Nb2 cells (75 x 10^6 at 1.5 x 10^6/ml) were treated for 4hr with 0 to 1000 nM okadaic acid (OA), or (B) for various times without (control) or with okadaic acid (OA, 1 μM). The activity of p70^{S6K} (S6K) and MAPK at Time 0 was 74 ± 8.1 and 43 ± 5.6 pmol phosphate transferred/mg protein/min., respectively. Results are expressed as the ratio of experimental to control and are representative of four separate, independent experiments. The activation of S6K and MAPK was significant at ≥10 nM OA, P M 0.05 (A) and at ≥30 min (B), $P < 0.05$.

The effect of PPase inhibition by OA on proximal Prl-like signals was examined next. The results illustrated that 1 μM OA (Fig. 2) persistently activated both MAPK and p70^{S6K}. Specifically, p70^{S6K} activity was increased ca. 4- and 8-fold at 2 and 4hr, respectively (Fig. 2, Panel A). This response was qualitatively and quantitatively different from that previously found with Prl, which activates p70^{S6K} at 2hr (2.3-fold) and slowly returns to basal activity at 4hr (9). MAPK activity, which is transiently stimulated by Prl (peak activity ca. 2.4-fold at 30 min, Ref. 9), is rapidly stimulated and sustained for 4hr in the presence of OA (Fig. 2, Panel B). The basal activity of these kinases is ca. 1/50 that of PPase (the specific activity of p70^{S6K} and MAPK is 75 and

43 pmol phosphate/mg protein/min, respectively vs. 2400 pmol phosphate/mg/min for PPase). These results suggest that OA inhibits the dephosphorylation of $p70^{S6K}$ and MAPK, thereby shifting the balance of these kinases to the phosphorylated (i.e., active) state.

We reported that several distal effects of Prl (i.e., protein and DNA synthesis as well as cell division) are coupled to the activation of MAPK and $p70^{S6K}$ (9, 14). To examine this relationship further, the effect of OA alone on these endpoints was determined. Okadaic acid (Fig. 3, Panel A) stimulates protein synthesis to an extent (2.1-fold) that is nearly equal to that found with 200 n*M* Prl (2.3-fold). The EC_{50} of OA is ca. 0.1 n*M*. This suggests a) that the inhibition of PPase activity amplifies the signal(s) employed by Prl to stimulate protein synthesis, or b) that OA affects additional signals that are independent of Prl or both.

In contrast to its stimulatory effect on kinase activity and protein synthesis, OA alone does not affect DNA synthesis or cell division. However, Prl-stimulated DNA synthesis and mitogenesis are potentiated by OA. Prolactin increases DNA synthesis 9-fold with an EC_{50} = 17 p*M* (Fig. 4) (10, 15). With both OA and Prl, the curve is shifted to the left, and the EC_{50} is reduced to 8 p*M*. The modest effect on cell doublings (2-fold) seen with Prl (4 p*M*) is increased to almost 5-fold with 1 n*M* OA (Fig. 5).

Discussion

The Nb2 lymphoma is well suited to examine Prl signal transmission because its response to Prl is receptor-mediated (14–16), involves a protein kinase cascade (5), and culminates in mitogenesis (9, 12). Okadaic acid, a potent inhibitor of PP2A/PP1 activity, was used in the present study to probe the influence of ser/thr PPase activity in Prl signaling. The effect of this agent is complex and differs according to the cell type, the concentration of inhibitor employed, and the duration of treatment (17). We observed that exposure of Nb2 cells to relatively high concentrations of OA (1 μ*M*) for 48hr had no deleterious effects on Nb2 cell viability and morphology. In many studies described here, n*M* concentrations of OA were used.

The present results demonstrate that OA mimics Prl action in stimulating MAPK and $p70^{S6K}$ activity and in augmenting protein synthesis. The effect on kinase activity is likely due to the inhibition of endogenous protein phosphatases (PP2A or PP1) because OA has no known direct effect on protein kinase activity (18). That treatment of Nb2 cells with OA alone results in a significant activation of MAPK, and $p70^{S6K}$ suggests there is a constant basal level of activity of these enzymes, that is attenuated by PP2A/PP1. The relatively high basal activity of PPase (Fig. 1) supports this suggestion. Of the two predominant PPases, PP2A and PP1, the former is ca. 100-times more sensitive to OA. However, OA cannot be used directly to determine which enzyme is acting on a particular phosphoprotein *in vivo*.

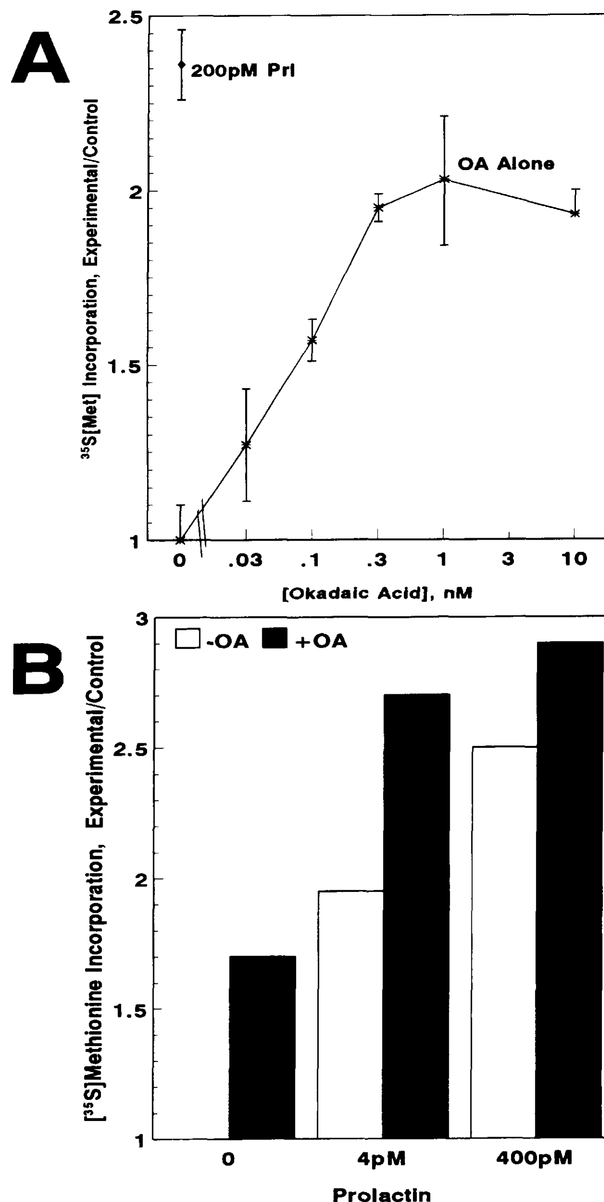


Figure 3. Effect of okadaic acid on protein synthesis in Nb2 cells. (A) Nb2 cell suspensions containing 2 μCi/ml [^{35}S]-methionine were incubated with vehicle (control), 200 p*M* prolactin (Prl) or okadaic acid (OA). Values are the mean ± SE of triplicate determinations and are representative of three separate, independent experiments. Protein synthesis is expressed as the ratio of experimental to control. The stimulatory effect of ≥0.1 n*M* OA was significant, $P < 0.02$. (B) Cells were treated as above except that 1 n*M*. OA and various concentrations of Prl were used. Values are the mean ± SE of triplicate determinations and are representative of at least three separate, independent experiments. The effect of OA on 4 and 400 p*M* Prl-mediated protein synthesis was significant, $P < 0.01$ and $P < 0.05$, respectively. Protein synthesis is expressed as the ratio of experimental to control. Control radioactivity was 133,388 ± 1806 CPM/10^6 cells.

The activation of MAPK and $p70^{S6K}$ by OA observed here (Fig. 2) is not a peculiarity of transformed Nb2 cells. Okadaic acid mimics insulin action in stimulating these kinases in freshly isolated epididymal fat pad adipocytes (18, 19). Okadaic acid also increases the activity and phosphorylation of $p70^{S6K}$ and MAPK in B-lymphocytes (20), 3T3

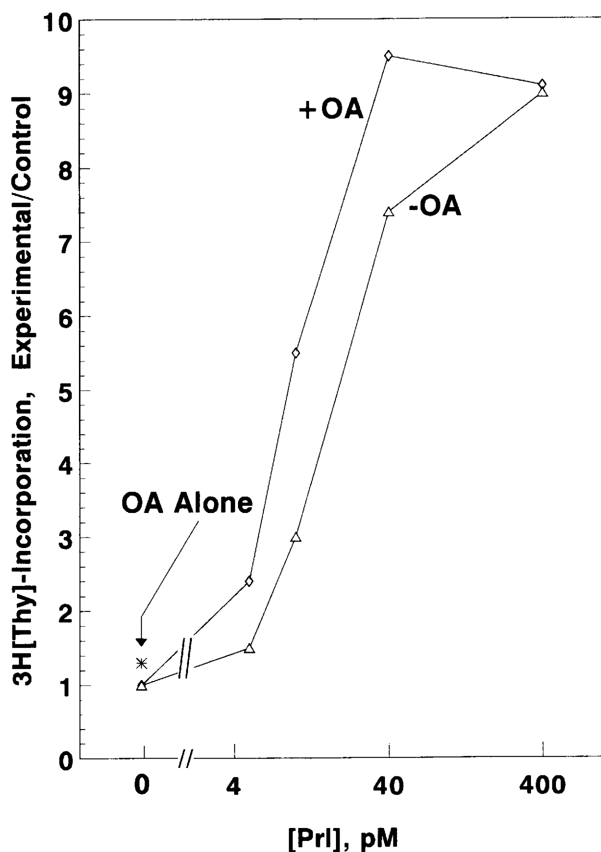


Figure 4. Effect of okadaic acid on prolactin-stimulated Nb2 DNA synthesis. Nb2 cells (0.5 x 10^6/ml) were incubated without (control, Ctrl) or with prolactin (Prl, 4–400 p*M*) and in the presence and absence of okadaic acid (OA, 1 n*M*). At 8hr of incubation, 1 µCi [^{3}H]thymidine/ml was added and acid-precipitable radioactivity was determined at 24hr. Radioactivity in 50 µl (25 x 10^3 cells) of the control group was 244,520 ± 18280 cpm/10^6 cells for triplicate determinations. Results are expressed as the ratio of experimental to control and are representative of three separate, independent experiments. The stimulatory effect of OA was significant from 4 and 40 p*M* Prl, $P < 0.02$.

cells (21), and human neutrophils (22). Lastly, OA also has been shown to activate MAPK in Jurkat cells (23). Together, these results suggest that basal cells possess a constant level of kinase activity that is actively suppressed by the action of PPases. The relatively high activity of PPase in Nb2 cells observed in the present study is consistent with this suggestion. We previously showed (9) that inhibition of MAPK and $p70^{S6K}$ greatly reduced Prl-mediated Nb2 protein synthesis. The present results support that observation and suggest that MAPK and $p70^{S6K}$ activation is necessary for increased protein synthesis. The mechanism by which OA increases protein synthesis is not known. However, of the two kinases affected by OA, $p70^{S6K}$ may be more directly linked to protein synthesis. This interpretation is consistent with recent results. It is thought that the phosphorylation state of PHAS-I is important in the regulation of protein synthesis initiation (24). Arnott *et al.* (25) reported that serum-induced PHAS-I phosphorylation is independent of the ERK1/ERK2 pathway in 3T3-L1 adipocytes. In addition, von Manteuffel *et al.* (26) demonstrated that 4E-BP1 phosphorylation is mediated by the FRAP-$p70^{S6K}$ pathway and independent of mitogen-activated protein kinase.

It was surprising that Nb2 cells failed to divide even though OA persistently stimulated MAPK and $p70^{S6K}$ activity and increased protein synthesis. In this context, it is interesting that Wilkie *et al.* (27) reported that the MAPK cascade is required but not sufficient for the mitogenic response to angiotensin II in vascular smooth muscle cells. It has been suggested that the duration of MAPK activation determines whether a mitogenic response will result (27–29). The present results and those of Wilkie *et al.,* who reported that phorbol myristate acetate, activated MAPK but did not stimulate mitogenesis of vascular smooth muscle cells do not support this notion. Results from other reports (17, 30) suggest that key phosphoproteins normally controlled by ser/thr PPases increase lymphocyte activation when kept in the phosphorylated state but require dephosphorylation for cell cycle progression. It has been shown that OA arrests the cell cycle of human leukemic cell lines HL-60 and U937 at G_2M and prevents progression through mitosis (31). Moreover, treatment of rodent fibroblasts with OA induces a pseudo-mitotic phenotype (32, 33). Little is known regarding the mechanism by which OA causes these

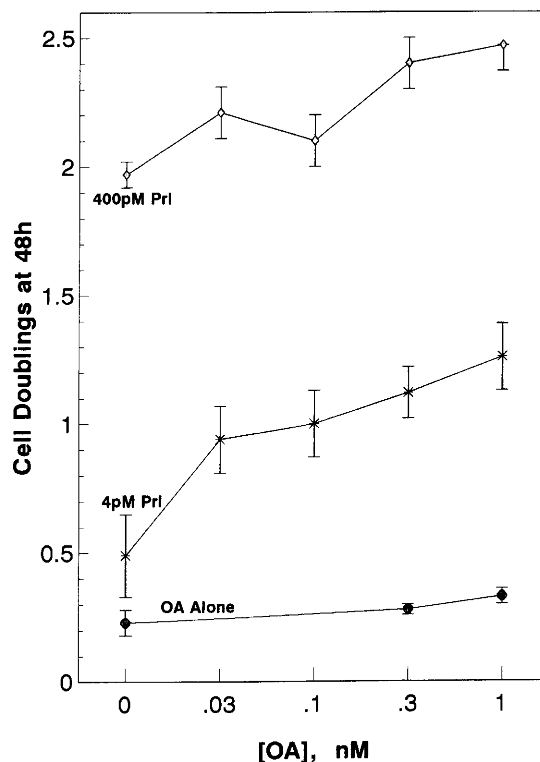


Figure 5. Effect of okadaic acid on prolactin-stimulated Nb2 mitogenesis. Nb2 cells (2 x 10^5/ml) were incubated without (control, Ctrl) or with prolactin (Prl) in the presence and absence of okadaic acid (OA, 0.1–1 n*M*). Cell number was determined at 48hr of incubation. Cell concentrations (per ml) were Ctrl, 25 ± 10^4: Prl-treated, 4 p*M* Prl, 31 ± 2 x 10^4 and 400 p*M* Prl, 108 ± 3 x 10^4. The number of cell doublings was determined by the formula: {N = log([cell]T_6[cell]T_6)/log2}. The results are expressed as the number of cell doublings at 48hr and are representative of three separate, independent experiments. OA (≥0.03 n*M*) significantly increased the mitogenic effect of 4 p*M* Prl, $P < 0.03$.

effects. However, premature activation of $p34^{cdc2K}$ by OA may induce entry into an M phase-like state (34, 35).

Taken together, we conclude that the activation of MAPK and $p70^{S6K}$ is necessary but not sufficient for Nb2 mitogenesis. Recently Wartmann *et al.* (36) reported that the activation of STAT5 (signal transducers and activator of transcription) by Prl is independent of the MAPK pathway. It is tempting to speculate that Prl-mediated mitogenesis requires the coordination of at least two separate and integrated signaling pathways (i.e., the cytoplasmic protein kinase cascade and the STAT pathway). The STAT pathway is insensitive to PP2A/PP1, bypasses MAPK and $p70^{S6K}$ and directly activates nuclear transcription factors (6, 16, 36–38). This possibility is currently under investigation.

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