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Induction of Ornithine Decarboxylase Activity by Growth and Differentiation Factors in FRTL-5 Cells

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Induction of ornithine decarboxylase has been correlated with the onset of cellular proliferation and cAMP production. Whether the resulting increases in polyamine levels are essential mediators of growth and/or differentiation or are merely incidental remains controversial. We have used FRTL-5 thyroid cells in culture to study the effects of three growth factors on ornithine decarboxylase activity. These factors [TSH, bovine calf serum, and 12-O-tetradecanoylphorbol-13-acetate (TPA)] are thought to act through different intracellular pathways. TSH stimulates cAMP production in thyroid cells, calf serum acts through ill-defined pathways to stimulate growth, and TPA is known to activate protein kinase C.

Bovine calf serum and TSH acted synergistically to induce ornithine decarboxylase activity. Activity was maximal when the phosphodiesterase inhibitor, methyl isobutyl xanthine, was included. Individually, neither serum nor TSH was a potent stimulator of the enzyme. Ornithine decarboxylase mRNA was apparent on Northern blots as a doublet following one hour of exposure to these agents. TPA did not stimulate ornithine decarboxylase activity and had an inhibitory effect on enzyme induction by TSH and serum. Difluoromethylornithine, a specific inhibitor of ornithine decarboxylase, inhibited growth induced by both TPA and TSH in putrescine-free medium. This effect was not apparent in medium containing 10^{-5}M putrescine. The data indicate that, although intracellular levels of cyclic AMP regulate ornithine decarboxylase activity, a component in serum is necessary for significant induction of this enzyme. Factors stimulating growth by non-cyclic AMP-dependent pathways may act without apparently stimulating this enzyme, although polyamines appear to be essential for their growth stimulatory effects.

INTRODUCTION

Ornithine decarboxylase (EC 4.1.1.17; L-ornithine carboxy-lyase; ODC) is the first and rate-limiting enzyme in the polyamine biosynthetic pathway [1,2]. Increases in enzyme activity and intracellular polyamine levels have been linked with cell growth and tumor promotion [2]. Various cAMP analogs, phosphodiesterase inhibitors, and hormones known to activate adenylate cyclase have also been shown to induce ODC in several cells and tissues [3], including the thyroid [4]. Whether elevated cAMP levels mediate trophic stimulation of ODC activity [5,6] depends on the tissue studied. Growth in an adrenocortical tumor cell line (Y1) is inhibited both by ACTH and 8 BrcAMP, yet both agents stimulate ODC activity [7]. Growth occurs in the presence of elevated levels of cAMP in a rat thyroid cell line (FRTL-5) [8], but the effects of cAMP and growth factors on ornithine decarboxylase activity have not been studied.

Abbreviations: CHO: Chinese hamster ovary DFMO: difluoromethylornithine DTT: dithiothreitol FRTL-5: Fischer rat thyroid line 5 percent serum MIX: methyl isobutyl xanthine ODC: ornithine decarboxylase TPA: 12-O-tetradecaroylphorbol-13-acetate TSH: thyroid stimulating hormone

The phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), a known tumor promoter, has been shown to cause increased ODC activity in a wide range of cells and tissues such as Chinese hamster ovary (CHO) cells [9,10] and mouse epidermal cell cultures [11]. Enzyme induction is postulated to proceed by a mechanism independent of cAMP-dependent kinase [9,10]. In thyroid cells we have shown that TPA exerts effects antagonistic to those mediated by elevations in cAMP [12].

In this present study, we examined three growth stimulators for their ability to induce ornithine decarboxylase activity in a cloned, normal line of TSH-dependent, functional rat thyroid cells (FRTL-5) [8,13]. In FRTL-5 cells, growth is dependent on the presence of insulin, thyroid stimulating hormone (TSH), and elevations in cAMP and bovine calf serum [8,14–16]. By themselves, none of these factors stimulates growth to any appreciable extent, but together there is synergy. The tumor promoter TPA also stimulates growth in this cell line but via a mechanism independent of cyclic AMP [12]. By using growth factors which act through discrete intracellular pathways, we hoped to define the role of ornithine decarboxylase induction in thyroid growth and function.

MATERIALS

Hormones and chemicals were purchased from the Sigma Chemical Corporation, St. Louis, MO. Media for cell culture were purchased from Gibco, Grand Island, NY. Difluoromethylornithine (DFMO) was kindly supplied by the Merrell Dow Research Institute, Cincinnati, OH. Omnifluor was supplied by New England Nuclear, and NCS tissue solubilizer came from Amersham. Mouse ornithine decarboxylase cDNA (pOD48) was kindly supplied by Dr. P. Coffino, University of California, San Francisco [17].

METHODS

Cell Culture

FRTL-5 cells were a generous gift from Dr. L. Kohn (N.I.H.) and the Interthyr Foundation and were cultured as described by Valente et al. [8] with the exception that the TSH concentration was maintained at 1 mU/ml. Cells are dependent on TSH for growth, and in its absence little or no growth is seen [8,14,15]. Cells were cultured for five days without TSH in 5 percent bovine calf serum and the five supplements described by Valente et al. [8], i.e., 10 μg/ml insulin, 10^{-8}M cortisol, 5 μg/mL transferrin, 10 ng/mL glycyl-L-histidyl-L-lysine acetate, and 10 ng/mL somatostatin. These five supplements are referred to as "5H" in articles describing these cells [8,13] and in the experiments presented in this paper. In all experiments presented, 5H was included in the medium. The combination of 5H with TSH is known as 6H.

ODC Assay

Cells were scraped from 9.6 cm^2 wells in 500 μL of ODC assay buffer [50 mM Tris HCl, 1 mM EDTA, 15 mM dithiothreitol (DTT) (pH 7.8)] and sonicated for 12 five-second periods on a Branson sonicator, speed 1. The homogenate was centrifuged at 27,000 g for 30 minutes. ODC activity was determined in 100 μL of supernatant, using the procedure of Kudlow et al. [7]. Assay mixture (200 μL) contained 50 mM Tris HCl (pH 7.8), 1.0 mM EDTA, 0.1 mM pyridoxal phosphate, 15 mM DTT, 0.4 mM L-ornithine, and 0.4 μCi of DL-[^{14}C]-ornithine with specificity activity 56 mCi/m mol, purchased from Amersham (Amersham Co., Arlington Heights, IL). On receipt,

isotope was mixed with an equal volume of 0.1 M HCl and evaporated to dryness on a rotary evaporator; this procedure removes a volatile contaminant [4] which gives high backgrounds in the ODC assays. The isotope was suspended in the original volume in 0.01 M HCl.

[Methyl-^{3}H]Thymidine Incorporation Assay

Cells were incubated in medium containing the five supplements, 5 percent calf serum, and 1 μCi/well [methyl-^{3}H]thymidine, specific activity 40–60 Ci/m mol (Amersham), and the supplements as noted for 48 hours. Experimental details for individual experiments are given in the figure legends. Experiments were terminated by removing the medium and washing the cell layer with ice-cold Hank's balanced salt solution. Cells were removed from the wells with 200 μL 0.1 M NaOH and transferred to centrifuge tubes. Wells were rinsed twice with 200 μL 0.1 M NaOH and washings were transferred to the tubes. The solubilized cells were incubated with 2.5 mL 6 percent trichloracetic acid for 20 minutes at 4°C. Tubes were centrifuged at 2,000 *g* for ten minutes in the cold. The precipitates remaining after aspiration of the supernatant were washed with 2 mL 6 percent trichloracetic acid and centrifuged again. Precipitates were dissolved in 400 μL NCS solubilizer and counted in 10 mL scintillation fluid containing 0.4 percent Omnifluor in toluene. Samples were counted in a Searle Mark III liquid scintillation counter with a dpm accessory.

DNA was also measured, using a diphenylamine colorimetric assay as described by Valente et al. [8].

Northern Blots

Total RNA was isolated from FRTL-5 cells by the method of Chirgwin et al. [18] and 30 μg electrophoresed in 0.9 percent agarose gels containing formaldehyde, as described by Maniatis et al. [19]. Following Northern transfer to nitrocellulose membrane, ODC mRNA was identified, using radiolabeled mouse ornithine decarboxylase cDNA (pOD48) kindly supplied by Dr. P. Coffino, University of California, San Francisco. The 1.6 kilobase insert [17] was purified by electroelution from agarose gels and labeled to a specific activity of 10^{-8}–10^{-9} cpm ^{32}P per μg DNA by the procedure described by Feinberg and Vogelstein [20]. Pre-hybridization and hybridization were performed as described by Shackleford and Varmus [21]. Bound radioactivity on the blots was detected by autoradiography at −70°C, using Kodak X-Omat AR film.

RESULTS

The effects of TSH and serum on ODC activity in FRTL-5 cells are shown in Table 1. Cultures were incubated for six hours in the conditions as noted. Results are expressed as dpm $^{14}CO_2$ released from 50 μL cell lysate. The assay blank which contained all the components of the assay mixture except protein gave a reading of 33 ± 1. Cells incubated in 5H alone showed no increased activity over the assay control blank. Bovine calf serum, itself a modest growth factor in the FRTL-5 cells [14], failed to stimulate ODC at 5 percent concentration and showed only a small increase at 30 percent concentration. In the absence of serum, using a wide range of TSH concentrations and incubation times, stimulation of ODC activity was poor. With TSH, however, calf serum acted synergistically to induce ODC activity in the FRTL-5 cells. By increasing the serum concentration from 5 percent to 30 percent, a further increase in ODC activity was seen in cells incubated for six hours in 1 mU per mL TSH.

TABLE 1
Synergism between Serum and TSH on Ornithine Decarboxylase Activity in FRTL-5 Cells

	dpm CO_2 Released		
	(−) CS	(+) 5% CS	(+) 30% CS
5H	35 + 0	34 + 2	53 + 10
5H + TSH	74 + 6	203 + 66	364 + 10

Assay blank = 33 ± 2. Mean ± SD $n = 4$

Cells were taken out of bovine calf serum for three hours before the start of the experiment. Incubations in the conditions as noted were continued for six hours before lysis of the cells and determination of ODC activity.

Maximal ODC activity required the presence of the phosphodiesterase inhibitor, 0.5 mM methyl isobutyl xanthine (MIX). Cells treated with TSH and 0.5 mM MIX for six hours had almost three times the ODC activity of cells incubated in TSH alone, as shown in Fig. 1. Cells incubated in 5H + 5 percent bovine calf serum showed no significant increase in activity over assay background activity of 48 ± 6.

A 24-hour time course of ODC induction following the addition of TSH and 30 percent bovine calf serum is shown in Fig. 2. A biphasic curve with peaks at four and 12 hours was seen, using the optimal conditions for ODC activity determined above. Experiments examining the time course of enzyme activity over shorter incubation periods showed no detectable enzyme activity before four hours and optimal activity at five to six hours. Experiments examining ODC activity induced by TSH or MIX independently in the presence of 5H and 30 percent bovine calf serum also showed this biphasic curve of induction of ODC activity.

The time course of induction of ODC mRNA is shown in Fig. 3. At the start of the experiment, cells in 5H and serum produced no detectable level of ODC; this was the

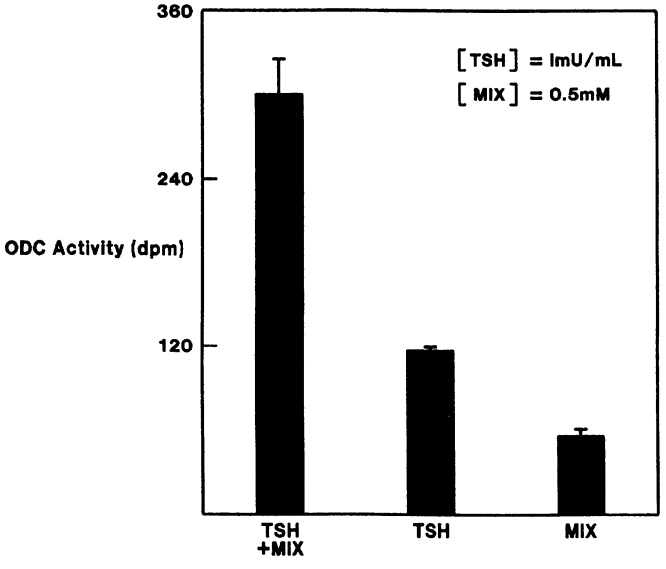

FIG. 1. Interaction between TSH and MIX on ODC activity in FRTL-5 cells. One mU/ml TSH and 0.5 mM MIX were added to the cells as noted. ODC activity was determined after six hours, using the protocol described in Methods.

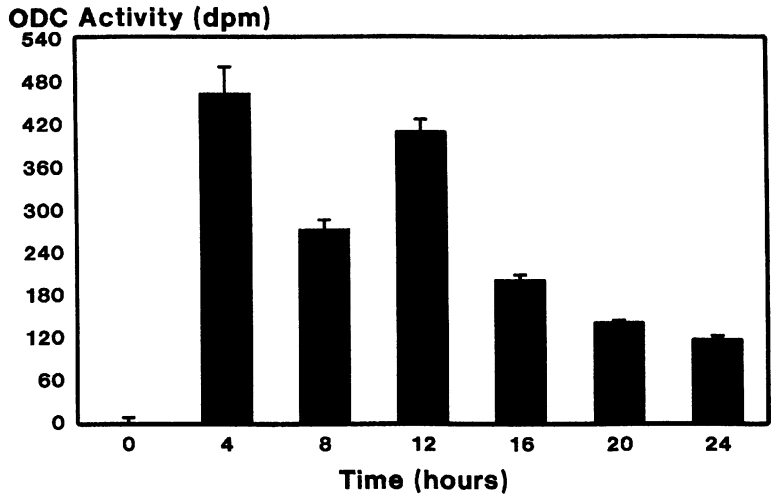

FIG. 2. Time course of ODC induction following TSH and MIX addition in the presence of 30 percent calf serum. Cells were assayed for ODC activity as described in the methods at the time noted after addition of 1 mU/ml TSH and 0.5 mM MIX.

control lane. One hour following media change to TSH, serum, MIX, and 5H, ODC mRNA was detectable. The level remained high at two hours but started to fall from this point onward. Detectable levels of ODC mRNA were, however, present at six hours following TSH and MIX addition.

The effect of TPA and TSH on [methyl-^{3}H]thymidine incorporation is shown in Table 2. In the absence of TSH, TPA (10^{-7}M) caused a tenfold increase in thymidine incorporation. This increase in DNA synthesis was not altered significantly by the addition of TSH. In the absence of TPA, TSH caused a dose-dependent increase in [methyl-^{3}H]thymidine incorporation with a 14-fold increase at a concentration of 1 mU per ml. The addition of TPA did not significantly inhibit the stimulation of DNA synthesis by TSH. Thus both TSH and TPA appear to increase thymidine incorporation but do not act in an additive or inhibitory manner in combination.

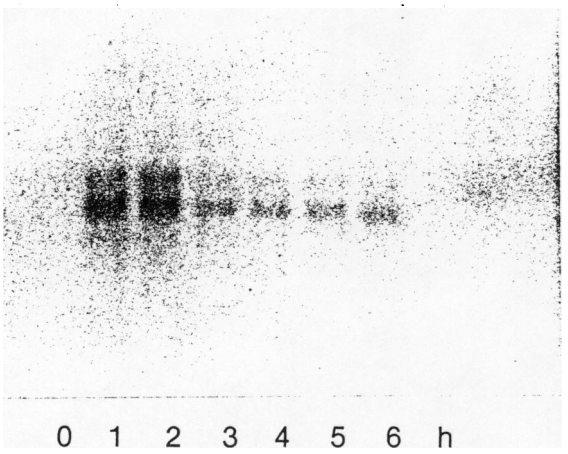

FIG. 3. Time course of ODC mRNA induction by 1 mU per mL TSH, 0.5 mM MIX, and 5 percent bovine calf serum in FRTL-5 cells. Equal amounts of total RNA were loaded per well, and, following electrophoresis, transferred to nitrocellulose, and probed with radiolabeled ODC cDNA.

TABLE 2
Effect of TPA on [Methyl^{3}H]Thymidine Incorporation in the Presence of TSH in FRTL-5 Cells

	dpm × 10^{-5} ± S.E.M.	
[TSH] per mL	−TPA	+TPA
0	0.23 ± 0.01	2.40 ± 0.56
10 μU	1.11 ± 0.11	2.42 ± 0.38
100 μU	2.62 ± 0.24	3.16 ± 0.40
1 mU	3.32 ± 0.45	2.70 ± 0.25

Cells were grown as described in Materials and Methods and assayed for radiolabeled thymidine uptake into acid-precipitable radioactivity, using the procedure described in Methods.

The effect of TPA on ODC activity is shown in Fig. 4. TPA alone consistently failed to stimulate ODC activity in the FRTL-5 cells following a six-hour incubation; however, 10^{-8}M TPA inhibited TSH-induced ODC activity in the cells. TPA caused a 63 percent reduction in ODC activity compared to the cells incubated in TSH alone. TPA did not alter the time course for maximal enzyme induction by TSH. The inhibitory effect was demonstrated at all time points examined, up to seven hours.

The effect of difluoromethylornithine (5 mM) and putrescine (10 μM) on ODC activity stimulated by TSH, MIX, and serum is shown in Fig. 5. Incubation in 10 μM putrescine, a concentration ten times that normally contained in this medium, inhibited ODC activity. Exogenous putrescine has been shown to cause a rapid decline in ODC activity because of an increased rate of degradation and decreased rate of synthesis of the enzyme [1,2]. Difluoromethylornithine (DFMO), an enzyme-activated irreversible inhibitor of ODC, was an even more potent inhibitor of enzyme activity.

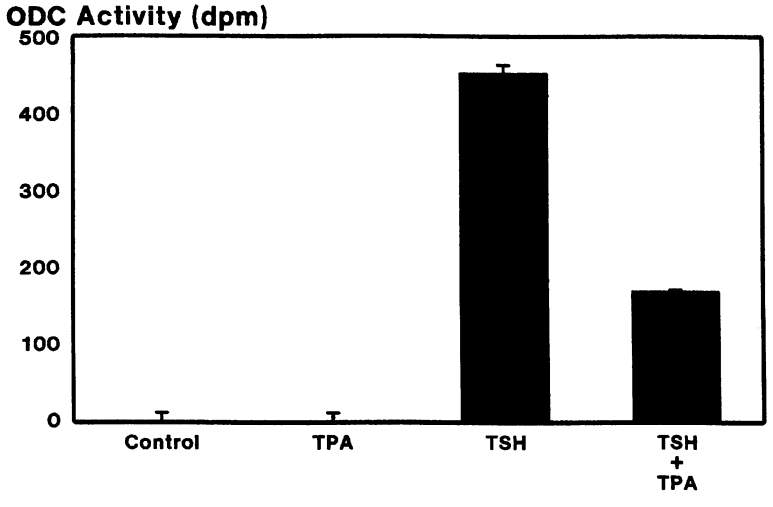

FIG. 4. Effect of TPA on TSH-induced ODC activity in FRTL-5 cells. 10^{-8}M TPA, 1 mU/ml TSH were added to the cells as indicated. ODC activity was determined after six hours, as described in Methods.

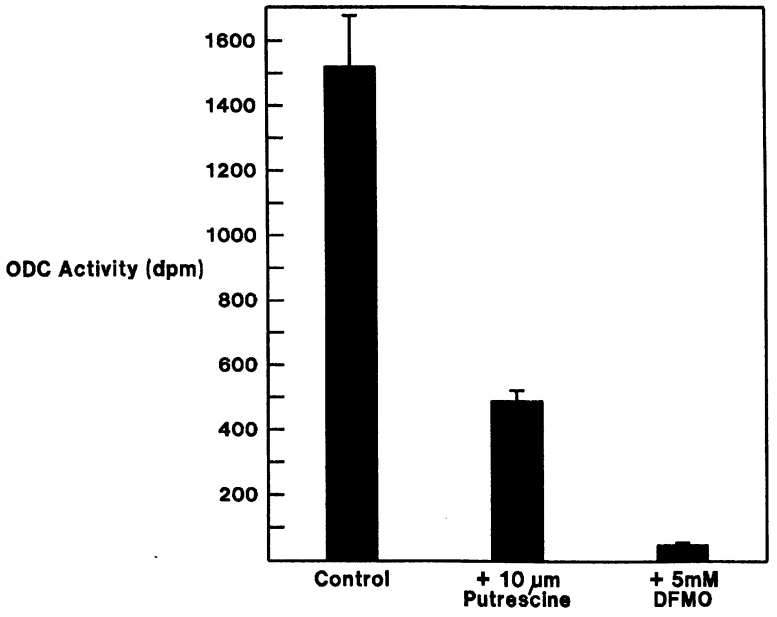

FIG. 5. Effect of 5 mM DFMO and 10^{-5}M putrescine on ODC activity. Cells were incubated in putrescine-free medium in 5H and dialysed calf serum for seven days before the start of the experiment. DFMO and putrescine were added to the incubation medium at the same time as 0.5 mM MIX and 1 mU per mL TSH. The experiment was terminated after six hours.

The effect of 5 mM DFMO and 10 μM putrescine on thymidine incorporation in FRTL-5 cells cultured under a variety of conditions is shown in Table 3. Cells incubated with TSH and 5 percent calf serum showed a modest stimulation of thymidine incorporation. This result was not inhibited by 5 mM DFMO when putrescine was included in the incubation, but incorporation was inhibited in the absence of putrescine. Stimulation by 5H in the presence of serum was comparable to

TABLE 3
Effect of DFMO and Putrescine on Methyl [^{3}H]Thymidine Incorporation into FRTL-5 Cells Stimulated with Various Agents

	− Putrescine		dpm × 10^{-3}	+ Putrescine	
	− DFMO	+ DFMO		− DFMO	+ DFMO
CS	3.5 ± 0.6	2.2 ± 0.5		0.7 ± 0.2	1.3 ± 0.9
TSH + CS	10.2 ± 1.7	5.1 ± 2.1		3.5 ± 0.7	3.5 ± 1.2
5H + CS	11.8 ± 4.4	7.6 ± 1.8		6.7 ± 4.9	4.0 ± 0.5
5H + CS + MIX	51.5 ± 8.4	24.6 ± 0.9		21.8 ± 11.4	36.3 ± 10.8
5H + CS + TPA	28.0 ± 5.3	9.4 ± 2.5		13.2 ± 2.6	13.9 ± 3.1

Mean ± SD $n = 4$

Following a four-day incubation in putrescine-free medium, putrescine (10^{-5} M) and DFMO (5 mM) were added as noted. Four days later, incubation medium was changed from 6H to 5H medium and DFMO and putrescine again replaced. Four days after that, cells were incubated in fresh putrescine-free medium with the additions as noted and 1 μCi/mL tritiated thymidine. The experiment was terminated after three days in this medium.

TABLE 4
Effect of DFMO on Growth Factor-Induced DNA Synthesis

	μg DNA	
	− DFMO	+ DFMO
5H	3.1 ± 0.7	2.7 ± 0.1
5H + TPA (10^{-7} M)	5.8 ± 0.3	2.9 ± 0.2
6H	12.1 ± 0.7	5.7 ± 0.5
6H + TPA	12.3 ± 0.2	6.1 ± 0.8

Mean ± SD $n = 3$

Cells were incubated in putrescine-free, 5H medium with 5 percent dialysed calf serum for five days before the start of the experiment, with one medium change during this period. Cells were incubated in fresh medium containing 5 percent dialysed calf serum and the conditions as noted for four days. DFMO was used at a concentration of 5 mM and TSH at 1 mU per mL. DNA was assayed using the diphenylamine colorimetric assay [7].

that of TSH and was inhibited in the presence of DMFO and in the absence of putrescine. The phosphodiesterase inhibitor, MIX, a strong stimulator of thymidine incorporation in the presence of 5H and 5 percent calf serum [16], also showed reduced levels of radiolabeled thymidine incorporation when incubated with DFMO in the absence of exogenous putrescine. The effects of TPA were similarly affected.

The effect of DFMO on growth factor-induced DNA synthesis is shown in Table 4. In these experiments, 5H and 5 percent bovine calf serum were included. Growth stimulation by TPA was inhibited completely by 5 mM DFMO. Growth stimulation by TSH (6H) and the combination of TPA with TSH was inhibited by 75 percent over controls.

DISCUSSION

TSH stimulates both growth and intracellular cAMP accumulation in the FRTL-5 cell line [8]. If indeed ODC induction by trophic stimuli is mediated by cAMP, treatment of these cells with thyrotropin should result in a significant increase in ODC activity. Calf serum has also been shown to stimulate growth in FRTL-5 [14]. In this study we found that maximum induction of ODC required the presence of both TSH and calf serum, acting in a synergistic manner; however, cyclic AMP is clearly a powerful stimulator because addition of a phosphodiesterase inhibitor enhances the effects of TSH and serum.

In response to trophic hormone administration, ODC induction is thought to exhibit a remarkably constant pattern of expression [3]. The time course of induction that we have shown in FRTL-5 cells correlates well with findings from the *in vitro* stimulation of rat thyroid by TSH [4,22]. ODC activity during the cell cycle is thought to be precisely regulated and expressed as a biphasic or double peak of activity, in late G_1 and G_2, respectively. This phenomenon has been demonstrated in various cells in culture [1,23]. The second increase in ODC activity shown in this report may represent the onset of late G_1, just prior to DNA synthesis, which begins approximately 16 hours following TSH stimulation of quiescent FRTL-5 cells. Increased levels of ODC mRNA were present at least three hours before there was a detectable increase in

enzyme activity. The delay in appearance of enzyme activity may be due to post-translational processing of the protein.

TPA also stimulated thymidine incorporation in this clonal line but did not stimulate ODC activity above background at all incubation times. TPA also inhibited the induction of ODC by TSH and bovine calf serum but did not reduce TSH-induced thymidine incorporation. These data provide another example of discrepant effects of growth factors on DNA synthesis and ODC stimulation. The growth stimulatory effects of TPA may be mediated through a non-cAMP-dependent pathway as described by Valente et al. [8]. TPA is known to activate protein kinase C, which may play a fundamental role in regulating cellular proliferation [24,25]. Possibly the inhibitory effects of TPA on TSH-induced ODC activity may be due to a reduction in cAMP produced following TSH action. Recent data show that phorbol ester may phosphorylate adenylate cyclase [26], and in rat osteoblasts, van Leeuwen et al. [27] showed that pre-incubation in TPA resulted in a dose-dependent inhibition of parathyroid hormone-stimulated cyclic AMP production. TPA also reduced forskolin-stimulated ODC activity in these cells but not PTH-stimulated ODC. In sheep thyroid cells in culture, we found that incubation in TPA or EGF with TSH reduced the accumulation of cyclic AMP [28]. In the preliminary studies, however, we have not been able to show a statistically significant reduction in cyclic AMP production in cells pre-treated with TPA for six hours or 24 hours (data not shown).

Data using DFMO show that its effects are prevented by addition of exogenous putrescine to the incubation medium. The experiments also show that both TSH- and TPA-stimulated growth are inhibited by DFMO. Although inhibition of the enzyme by DFMO does inhibit TPA-mediated growth, this process may occur because there are inadequate basal levels of polyamines to support growth rather than a requirement for elevated levels. There may be a minimum requirement for polyamines that does not require high levels of induction of the enzyme. Although ODC levels rise in response to TSH, our data show that protein kinase C activation results in reduced levels of enzyme activity while growth is stimulated. Further investigation may reveal that the FRTL-5 is an example of a cell line in which ODC induction is not necessary for subsequent cell growth.

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