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Effect of Okadaic Acid and Calyculin-A, Two Protein Phosphatase Inhibitors, on Thyrotropin-Stimulated Triiodothyronine Secretion in Cultured Sheep Thyroid Cells

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We have studied the effect of two protein phosphatase inhibitors on thyrotropin (TSH)-stimulated triiodothyronine (T_3) production by sheep thyroid cells grown in primary culture. Incubation of sheep thyrocytes with okadaic acid (OA) and calyculin-A (CL-A), two potent inhibitors of type 1 (PP1) and type 2A (PP2A) protein phosphatases, resulted in an increase of TSH-stimulated T_3 production. This effect was detected using concentrations as low as 0.1 p*M* with OA and 1 f*M* with CL-A. An inhibitory effect on T_3 production, due to cellular death, was observed with 6 n*M* OA and 1 n*M* CL-A. In the absence of TSH, OA or CL-A had no effect on T_3 production by thyrocytes. Forskoline (10 μ*M*), an activator of adenylate cyclase, increased the basal and TSH-stimulated T_3 release by sheep thyroid cells; this effect was increased by OA in cells grown in the basal state but not in the presence of TSH. These results suggest that the marine toxins OA and CL-A, two potent inhibitors of PP-1 and PP-2A, have significant stimulatory effects on T_3 secretion promoted by TSH and FK. These observations indicate that these proteins could be important mediators of thyroid hormone production.

Key Words: Okadaic acid; calyculin A; triiodothyronine; protein phosphatases

Introduction

Thyroid-stimulating hormone (TSH) is an important regulator of thyroid hormone production, although other agents such as growth factors also appear to exert important influences on hormone production. TSH and other regulatory compounds are thought to mediate their effects by stimulation of specific receptors, which in turn activate one or more of the second-messenger systems found in the thyrocyte. Three second-messenger systems have been identified in the thyrocyte: cyclic adenosine monophosphate (cAMP), Ca^{2+}-phosphoinositol (Ca^{2+}-PI), and tyrosine kinase cascades *(1)*.

In humans, TSH activates both adenylyl cyclase and phospholipase C pathways *(2)*, leading to the activation of cAMP-dependent proteinkinase A, calcium/phosphatidylinositol- dependent protein kinase C, and calcium/calmodulin-dependent protein kinases. In FRTL-5 thyrocytes, TSH rapidly stimulates inositol triephosphate formation *(3)*. Eggo et al. *(4)* have reported that acute production of inositol trisphosphate does not appear to be involved in the mechanism of action of TSH in stimulating sheep thyroid secretion, but Ca^{2+} mobilization is implicated. The kinases, in turn, induce increases in phosphorylation in serine and threonine residues in a number of proteins *(5,6)*.

Protein phosphorylation may be involved in the activation of numerous processes in thyroid cells, including increased iodide uptake and H_2O_2 generation, stimulation of thyroid peroxidase and thyroglobulin expression, and activation of endocytosis of thyroglobulin *(7)*. The protein phosphorylation state is not only dependent on the activity of protein kinases, but also on the action of protein phosphatases. Although numerous observations indicate the importance of protein kinase activation in thyroid cells *(1,2,8)*, the role of protein phosphatases in TSH-stimulated thyroid hormone production is poorly characterized.

In this study we used cultured sheep thyrocytes and two structurally different protein phosphatase inhibitors, okadaic acid (OA) and calyculin-A (CL-A), to investigate the role of phosphoprotein phosphatases in TSH-stimulated thyroid hormone production. OA is a polyether fatty acid produced by dinoflagellates and was originally isolated from the marine sponge *Halichondria okadaii*. The compound is a cause of diarrhetic shellfish poisoning and is a potent tumor promoter *(9,10)*. OA is a very potent inhibitor of type 1 (PP1) and type 2A (PP2A) ser/thr protein phosphatase, two

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of the four major protein phosphatases in the cytosol of mammalian cells that dephosphorylate serine and threonine residues *(11)*. CL-A, a marine toxin isolated from the sponge *Discoderma calyx*, is also a tumor promoter and an inhibitor of PP1 and PP2A, but is 10- to 100-fold more potent than OA at inhibiting PP1 in tissue extracts *(12)*.

Protein phosphatase inhibitors may modify protein phosphorylation induced by a number of signaling pathways. We have, therefore, also studied the effects of forskolin, an activator of adenylate cyclase.

Results

Effect of Protein Phosphatase Inhibitors on Thyroid Hormone Secretion

TSH produced approximately a fivefold stimulation of triiodothyronine (T_3) production by sheep thyrocytes over a 4-d incubation period. Figure 1 shows the effect of OA on T_3 production in sheep thyrocytes incubated in the presence or absence of TSH. OA had no effect on T_3 production in the absence of TSH. In TSH-stimulated cells, T_3 production was significantly increased by 20–40% when OA was added at concentrations ranging from 0.1 p*M* to 1 n*M*. A marked inhibition of T_3 production was found when OA was added at 6 n*M* (with or without TSH), but this appeared to result from cellular death, which was measured by microscopic examination (results not shown).

Figure 2 presents the effect of CL-A on basal and TSH-stimulated T_3 secretion. Incubation of sheep thyrocytes with CL-A at doses ranging from 1 f*M* to 1 n*M* had no significant effect on T_3 secretion in the absence of TSH. When TSH was present, however, CL-A addition at doses of 1 f*M* to 0.1 n*M* stimulated T_3 production by 50–70%. Incubation with CL-A at 1 n*M* significantly decreased (50% inhibition) T_3 levels; this could be attributed to cellular death.

Effect of Forskolin and OA on T_3 Secretion

Figure 3 presents the effects of forskolin (10 μ*M*) and OA (0.1 n*M*) on T_3 production in the presence or absence of TSH. In cells grown in the absence of TSH, forskolin produced approximately a threefold increase in T_3 production. The inclusion of OA with forskolin into the culture medium produced more than a fourfold increase in T_3 production, whereas OA added alone had no effect on T_3 production TSH produced a four- to fivefold increase in T_3 production and the addition of forskolin was able to produce a further small but significant increase in T_3 production above that observed in cells treated with TSH alone. As in the experiments described above, OA significantly increased T_3 production in the presence of TSH, but OA addition had no stimulatory effect on T_3 production when both forskolin and TSH were present.

Discussion

We have studied the effects of protein phosphatase inhibitors on T_3 production in sheep thyrocytes maintained in primary culture. Thyrocytes grown in this culture system produce T_3 by *de novo* synthesis if the optimal concentrations of 10 μ*M* KI and 1 mU/mL TSH are present in the culture medium *(13)*. Sheep thyroid does not express type I iodothyronine deiodinase, and, as such, none of the T_3 produced by sheep thyrocytes arises from intracellular deiodination. In the absence of TSH, T_3 secretion into the culture medium is small and appears to arise both from a low rate of T_3 synthesis and a release of preformed endogenous thyroid hormone that is present in the thyrocytes when they are isolated from thyroid tissue. Thus, the data presented on cells incubated in the absence of TSH (the basal state) largely reflect the release of preformed hormone, whereas in TSH-treated cells, T_3 production largely arises from hormone synthesis *(13)*. Thyroxine is also synthesized by these cells but less efficiently than T_3 *(13)*.

The treatment of sheep thyroid cells with inhibitors of protein phosphatases had significant effects on TSH-stimulated T_3 production. Both OA and CL-A increased T_3 production, but CL-A was more potent than OA in this respect. Significant effects on T_3 production could be achieved with OA concentrations of 0.1 p*M*, and a similar effect was achieved with CL-A added at a concentration of 1 f*M*. These observations agree with previous reports that CL-A is far more potent than OA at inhibiting PP1 in tissue extracts *(14)*.

Essential steps in thyroid hormone formation are regulated by TSH using different second-messenger pathways that promote phosphorylation of different protein substrates *(15)*. Our results are consistent with the view that increased T_3 synthesis, signaled through the TSH receptor, involves protein phosphorylation and that endogenous phosphoprotein phosphatases play an important role in regulating thyroid hormone production.

At higher concentrations, we found that both OA and CL-A had inhibitory effects on T_3 production. This arose because of cytotoxicity which was achieved with an OA concentration of 6 n*M* and a CL-A concentration of 1 n*M*.

Forskolin increases intracellular cAMP levels *(16)* and, as we have shown (Fig. 3), leads to a marked stimulation of T_3 production in cells grown in the absence of TSH. Indeed, the addition of forskolin (10 μ*M*) stimulated T_3 production to levels similar to those found in TSH-treated cells, supporting the widely held view that the stimulatory actions of TSH on thyroid hormone production arise through the cAMP signaling pathway. In the absence of TSH, T_3 production induced by forskolin was further enhanced by the simultaneous addition of OA. These observations also suggest that the phosphoprotein phosphatases may play an important role in regulating thyroid hormone production signaled through the cAMP pathway. Maximal T_3 production was observed in cells treated with both TSH and forskolin, and in these cells no effect of OA could be observed. In contrast, the lack of effect of OA on cells simultaneously treated with TSH and FK probably reflects that protein phosphorylation levels were maximally enhanced by

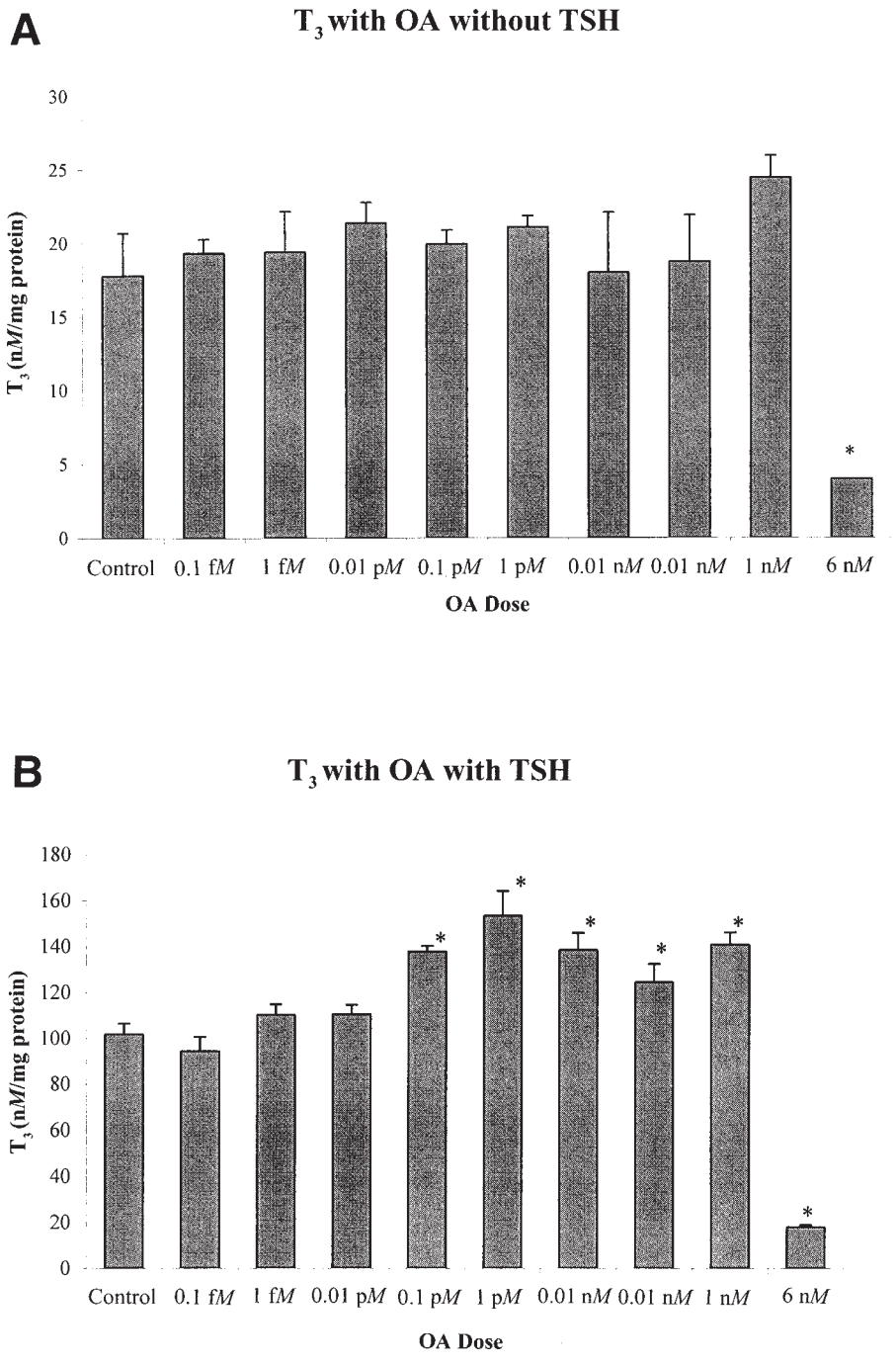

Fig 1. Effect of OA on basal **(A)** and TSH-stimulated **(B)** T_3 secretion in sheep thyroid cells. Thyrocytes (25×10^5 cells/well) were cultured as described under Materials and Methods with and without TSH (1 mU/mL) and increasing concentrations of OA as specified After 4 d in culture, the accumulated content of T_3 in the culture medium was assayed by radioimmunoassay (RIA). Data were expressed as means ± SEM of triplicate wells in a representative experiment conducted on three occasions. * $p < 0.05$ with respect to control levels.

both agents in cultured thyrocytes, thus counteracting the tonic inhibitory effect of cellular phosphoprotein phosphatase on T_3 secretion.

Métayé et al. *(17)* have observed that in human thyroid cells, PP1 and PP2 can regulate TSH-mediated cAMP production with OA or CL-A, showing a biphasic and dose-dependent inhibitory effect on cAMP accumulation. We have not investigated the effects of OA or CL-A on cAMP production by sheep thyrocytes, but our observation that the addition of OA in the presence of TSH and forskolin had no inhibitory effect on T_3 production suggests that any effects of OA on cAMP production must be minor.

Although our experiments were confined to the use of isolated cells, the marine toxins OA and CL-A have effects on all tissues including the pituitary. Michimata et al. *(18)* have observed that preincubation of anterior pituitaries with

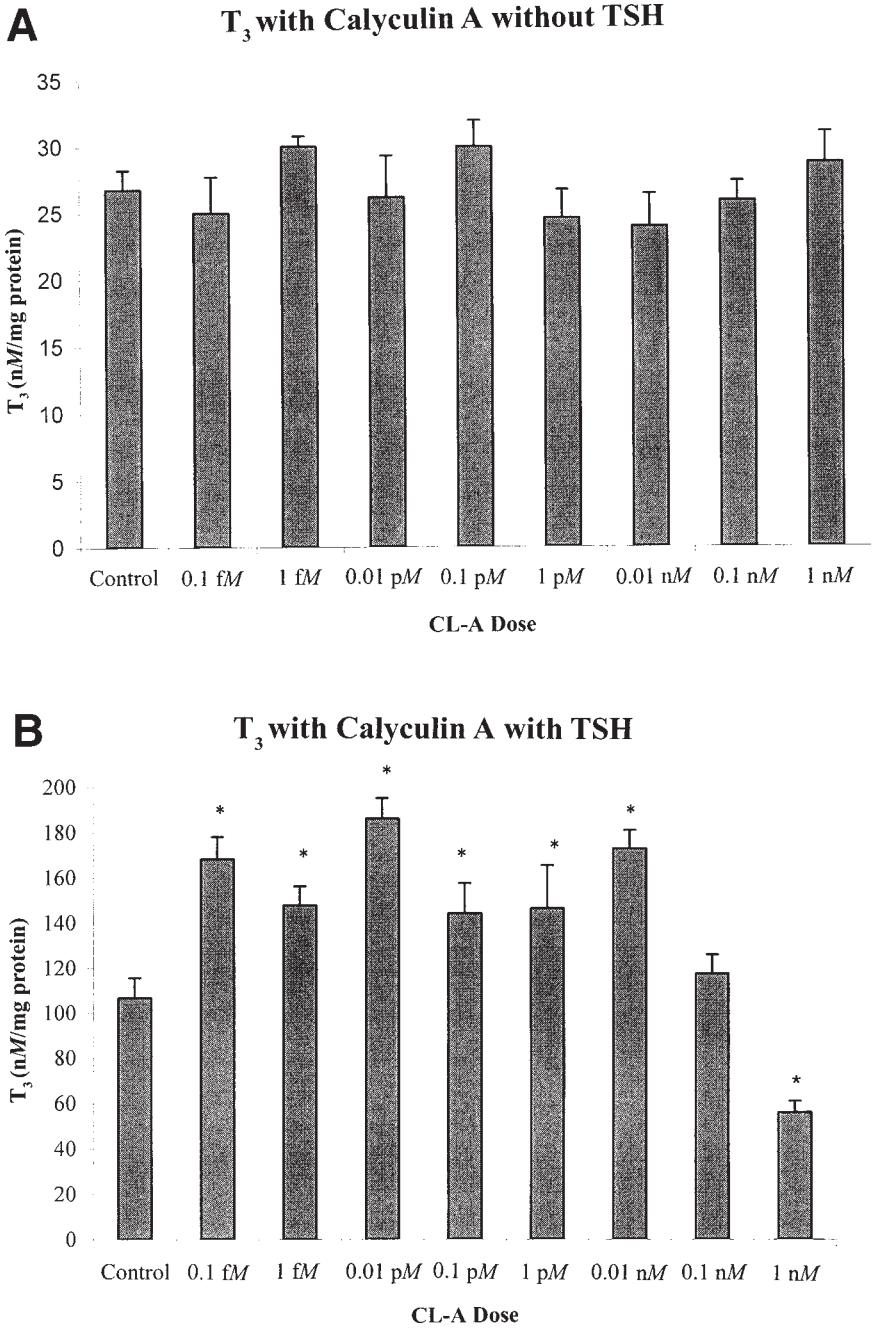

Fig 2. Effect of CL-A on basal **(A)** and TSH-stimulated **(B)** T_3 secretion by sheep thyroid cells. Thyrocytes (25×10^5 cells/well) were cultured as described in Materials and Methods with and without TSH (1 mU/mL) and increasing concentrations of CL-A as specified. After 4 d in culture, T_3 accumulated in the culture medium was assayed by RIA. Data were expressed as means ± SEM of triplicate wells in a representative experiment conducted on three occasions. * $p < 0.05$ with respect to control levels.

OA caused a time- and concentration-related decrease in a subsequent thyrotropin-releasing hormone–stimulated TSH secretion, whereas it did not cause any changes in basal secretion of TSH. It is thus possible that in vivo, the agents may also exert indirect effects on thyroid hormone synthesis through modifying plasma TSH.

In conclusion, our data have shown that the marine toxins OA and CL-A, two potent inhibitors of PP-1 and PP-2A, have significant stimulatory effects on T_3 secretion promoted by TSH and forskolin. These observations indicate that these proteins could be important mediators of thyroid hormone production

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and Earle's balanced salt solution (EBSS) were obtained from ICN Flow (Costa Mesa, CA). Collagenase was purchased

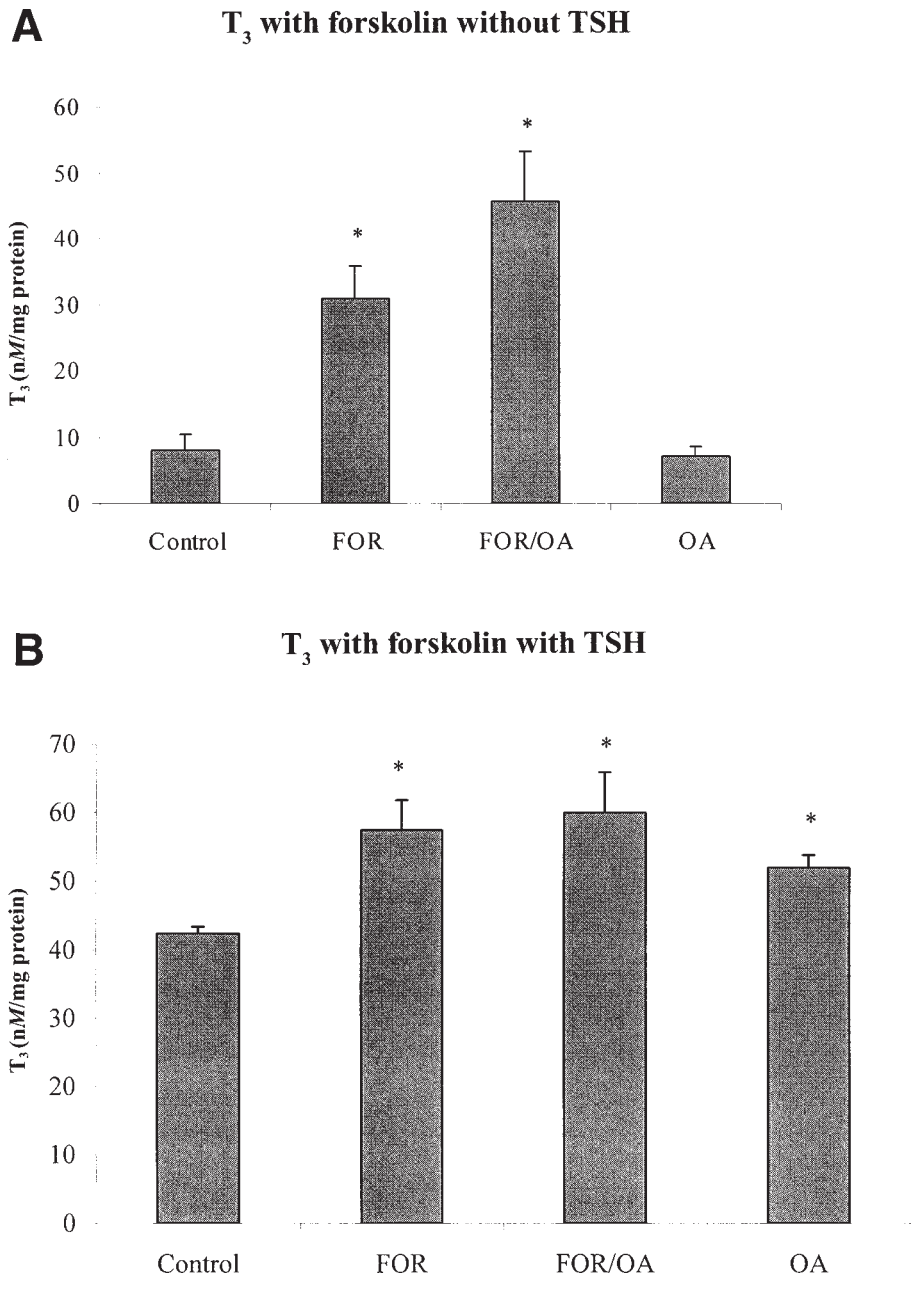

Fig. 3. Comparative effect OA and forskolin on T_3 secretion by sheep thyroid cells. Thyrocytes (25×10^5 cells/well) were cultured with and without TSH (1 mU/mL) in the absence or presence of OA (0.1 n*M*) or Forskoline (10 μ*M*). After 4 d in culture, T_3 accumulated in the culture medium was assayed by specific RIA. Results show the means ± SEM of triplicate wells in a representative experiment conducted on three occasions. * $p < 0.05$ with respect to control. FOR, forskolin.

from Worthington Biochemicals via Lorne Laboratories (Twyford, Beks, UK), and dispase was supplied by Boehringer Mannheim UK (Lewes, UK). TSH (NIBSC code 53/011) was supplied by the National Institute for Biological Standards and Controls (London, England), and culture plates were purchased from Merck via BDH (Poole, UK). Penicillin/streptomycin, amphotericin, and glutamine were obtained from NBL (Cramlington, UK). CL-A was obtained from RBI (Biogen Científica S L, Madrid, Spain). All other reagents including OA and forskolin were obtained from Sigma (Poole, UK).

Total T_3 concentration was determined by RIA using Amerlex M kits purchased from Ortho-Clinical, Amersham, UK. The detection limit (with a probability of 95%) was 0.15 n*M*. The intra- and interassay coefficients of variation were 37 and 49%, respectively.

Isolation and Culture of Sheep Thyrocytes

Sheep thyrocytes were isolated from normal tissue obtained within 30 min of death from animals in the local abattoir. The cells were isolated as described previously *(13)*. Briefly, the tissue was minced with scissors, and the

resulting fragments were then washed four times with EBSS and digested for 2 h in 50 mL of an enzyme cocktail containing dispase (5 mg/mL), trypsin (025%, w/v), and collagenase (1 mg/mL) in EBSS. Following digestion, 50 mL of DMEM containing 10% (w/v) fetal calf serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin, 25 μg/mL of amphotericin B, and added 2m*M*/L of glutamine and 10 μ*M*/L of KI was added and the mixture filtered through a 100-μm mesh gauze to remove undigested tissue. The resulting filtrate, containing released thyroid cells, was centrifuged at 1500 rpm for 5 min to pellet the thyrocytes and leave the majority of erythrocytes in suspension. The pellet was then resuspended in 50 mL of DMEM, and the cell yield was measured with a modified Neubauer hemocytometer. Finally, dispersed thyrocytes were plated out in DMEM into 24-well plates (16 mm diameter) at a density of 25×10^5 cells/well in 500 μL of medium. The cells were incubated at 37°C in an atmosphere of 5% CO_2 for 24 h before the start of any experiment.

Effect of Protein Phosphatase Inhibitors on T_3 Production by Sheep Thyrocytes

After 24 h, the medium was removed from sheep thyrocytes in monolayer culture, and the cells were washed with 1 mL EBSS (three times) to remove any dead cells and thyroid hormones released from viable cells during the first 24 h of culture. Fresh culture medium containing a fixed dose of KI (10 μ*M*/L) was added to all wells *(13)*. Cultures of thyrocytes were supplemented with TSH (1 mU/mL), as indicated. In some cultures, TSH was omitted and these were regarded as basal controls to study thyroid hormone synthesis and release in the basal state. All cultures were performed in triplicate and the T_3 released into the medium over 4 d of incubation was measured by RIA. Results were corrected for cell protein content determined using the Bradford dye-binding method *(9)* adapted for use on a Cobas Fara centrifugal analyzer (Roch Diagnostics, Welwyn Garden City, UK).

To study the effects of protein phosphatase inhibitors, cultures of thyrocytes were treated with OA or CL-A over a wide range of concentrations as specified in Results. In some experiments, the effects of OA on thyrocytes treated with or without forskolin (10 μ*M*) were also studied using the same 4-d incubation protocol.

Statistical Analysis

Analysis of variance and the Tukey-Kramer Multiple Comparisons test were used to analyze the data. Differences among groups of $p<0.05$ were considered significant.

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