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PHORBOL ESTERS AS PROBES OF THE REGULATION OF THYROTROPIN SECRETION

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12-O-Tetradecanoylphorbol 13-acetate and thyrotropin releasing hormone were compared as thyrotropin secretagogues in cultured rat pituitary cells. The maximal secretion evoked by the former was only half that evoked by the latter. A23187 plus 12-O-tetradecanoylphorbol 13-acetate together produced as large a response as thyrotropin releasing hormone. Triiodothyronine inhibited phorbol ester-induced thyrotropin secretion, and this inhibition required protein synthesis.

Regulation of TSH secretion plays a central role in thyroid hormone homeostasis. TRH appears to be the major physiologic TSH secretagogue, and T_3 the major inhibitor of TRH-induced TSH release. Recently, it has been shown that the binding of TRH to its receptor is followed within seconds by PI turnover (1,2). This leads to an increase in intracellular Ca^{++}, which appears to be a second messenger in TSH release. The precise manner in which an elevated cytosolic Ca^{++} concentration is coupled to secretion is unknown. However, the Ca^{++} and phospholipid dependent protein kinase (protein kinase C) is activated, and a number of protein phosphorylations occur (3,4).

The mechanism whereby T_3 inhibits TRH-induced TSH secretion also is not known. Protein synthesis inhibitors block this T_3

Abbreviations: BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; IP_3, inositol triphosphate; PDD, phorbol 12,13-didecanoate; PI, phosphatidylinositol; TPA, 12-O-tetradecanoylphorbol 13-acetate; TSH, thyrotropin; TRH, thyrotropin releasing hormone; T_3, 3,5,3'-triiodo-L-thyronine.

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effect (5). In addition, T_3 affects calcium metabolism in rat anterior pituitary cells superfused in vitro (6).

In the present investigation, the influences of TRH, T_3 and calcium on TSH secretion have been studied in vitro. Phorbol esters, which bind to and activate protein kinase C, have been used to study the role of this enzyme in TSH secretion.

MATERIALS AND METHODS

Materials. Male Sprague-Dawley rats, 125-250 g, were obtained from Zivic-Miller. Tissue culture media, dishes and antibiotics were purchased from Grand Island Biological Co. The following reagents were obtained from Sigma Chemical Co.: collagenase type II, trypsin type III, soy bean trypsin inhibitor, T_3, TRH, TPA, 4α-PDD, 4β-PDD, and deoxyribonuclease 1. Cycloheximide and A23187 were obtained from Calbiochem, and fibronectin from Collaborative Research. Stock solutions of phorbol esters (3 mM) and A23187 (10 mM) were prepared in DMSO and stored at -20C.

Cell dispersion and culture. Anterior pituitary cells were dispersed and plated into 96-well clusters (200,000 cells/well) as described previously (7), except that the serum was fetal bovine (heated to 56C, 30 min). The cells were cultured overnight at 37C in 5% CO_2. The media were then changed so that the serum (heated 56C, 30 min) was from hypothyroid rats (7). Experiments were performed after overnight incubation in this media.

TSH secretion. On the day of an experiment, cells were incubated in medium 199 with 25 mM $NaHCO_3$ and 12.5 mM HEPES. An initial 2 hour incubation was used to expose the cells to various T_3 concentrations (or control media). The media were removed and replaced with 0.15 ml/well of fresh media containing the same T_3 concentrations. Incubation was for another 25 min, after which 0.13 ml of media/well were removed. Each well received 0.13 ml of fresh media containing a potential secretagogue (TRH, phorbol ester, A23187, or control) plus the same concentration of T_3. After another 25 min incubation, 0.13 ml of media/well were harvested for TSH assay. This procedure was devised to minimize carryover of TSH from one incubation period to the next, and to minimize removal of cells with the media. All media for both 25 min incubations were preequilibrated at 37C in 5% CO_2. In all studies involving cycloheximide, this drug was present at 10^{-5} M during all 3 incubation periods, and the initial incubation was for 1 hour rather than 2. In all experiments, every condition was studied in quadruplicate wells.

TSH assay. The media that were harvested during the experiment were centrifuged at 4C at 400 g for 5 minutes. Fifty microliters of supernatant were placed into each of 2 tubes and assayed for TSH (radioimmunoassay kit provided by the National Pituitary Agency). Results are expressed as ng TSH secreted/well (mean ± SEM) during the final 25 min incubation containing the potential secretagogues. Net secretion refers to total TSH secretion minus basal. To convert ng TSH to microunits, multiply by 0.22.

RESULTS

TRH was a potent secretagogue of TSH, inducing a 4-fold increase over basal secretion (Fig. 1). The ED_{50} for TRH-induced TSH secretion was 2×10^{-9} M. TPA also stimulated TSH release (Fig. 1), with an ED_{50} of 10^{-8} M. A slight increase in TSH secretion was seen in the presence of 0.1% DMSO (present at the highest dose of TPA tested). This DMSO effect (30 ng TSH over basal secretion) was subtracted from the highest dose TPA data to obtain the results in Fig. 1. The maximal effect seen with TPA was significantly less than with TRH ($p=.002$, by t-test).

The TPA induced secretion was specific for those phorbol esters that are known to be tumor promoters and capable of activating protein kinase C. Thus, incubation with the tumor promoter 4β-PDD at 3×10^{-7} M increased TSH secretion in two experiments from 141 to 195 ng. In contrast, 4α-PDD, which is not a tumor promoter, did not stimulate TSH secretion (144 ng in the same 2 experiments).

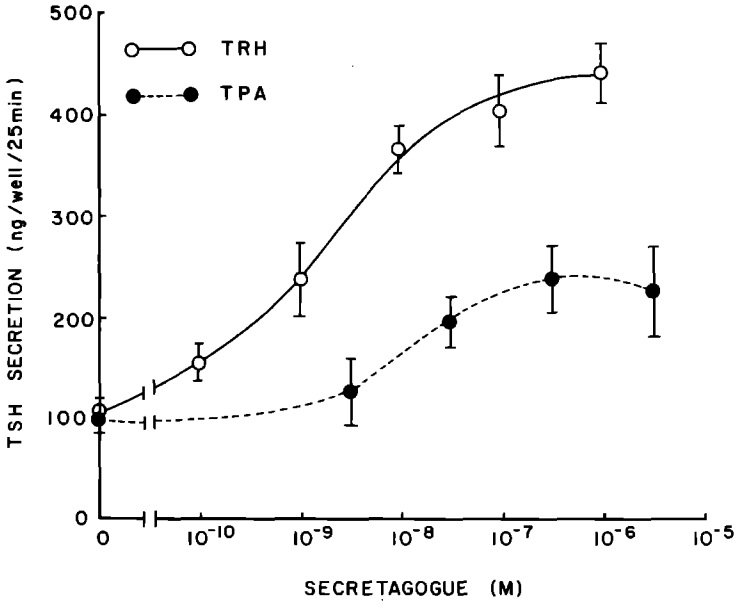

Fig. 1. Dose-response curves for TRH- and TPA-induced TSH secretion. The results of 6 experiments are plotted.

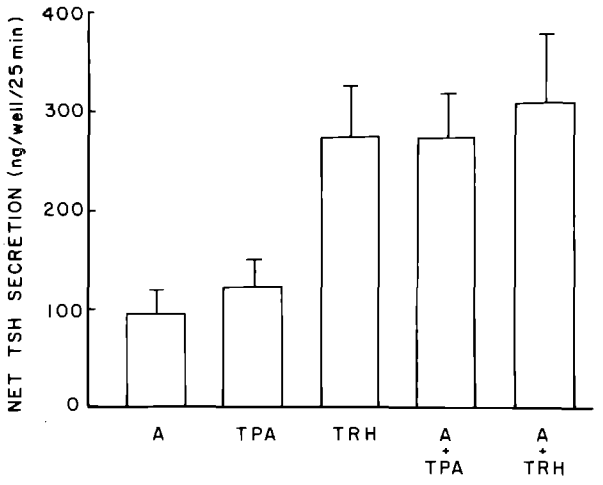

Fig. 2. Effects of 10^{-5} M A23187 (A), 3 x 10^{-7} M TPA, 10^{-6} M TRH, and combinations on TSH secretion. Basal secretion was 117 ± 15 ng. The results of 5 experiments are plotted.

The reason why TRH is more potent than TPA as a TSH secretagogue was explored. Both agents are capable of activating protein kinase C, but only TRH can increase cytosolic Ca^{++}. Therefore, TPA was studied in concert with the calcium ionophore A23187. As seen in Fig. 2, 10^{-5} M A23187 was a modest TSH secretagogue. The combination of TPA plus A23187 was more potent than either agent alone ($p=.02$, by Newman-Keuls test), and equally potent as TRH alone. The combination of TRH plus A23187 did not evoke more TSH secretion than TRH alone ($p>.5$).

The interaction of TPA with TRH also was evaluated. In 7 experiments using maximally effective doses of both secretagogues (3 x 10^{-7} M and 10^{-6} M, respectively), the combination of TPA plus TRH evoked more TSH secretion than TRH alone. Net secretion was 360 ± 34 ng for TPA plus TRH, and 285 ± 40 ng for TRH alone ($p=.01$, by paired t-test). In contrast, in 2 experiments, when a dose of TRH that is approximately an ED_{50} (10^{-9} M) was studied plus and minus a low dose of TPA (3 x 10^{-9} M), TRH plus TPA was not a more effective secretagogue than TRH alone (132 versus 153 ng net, respectively).

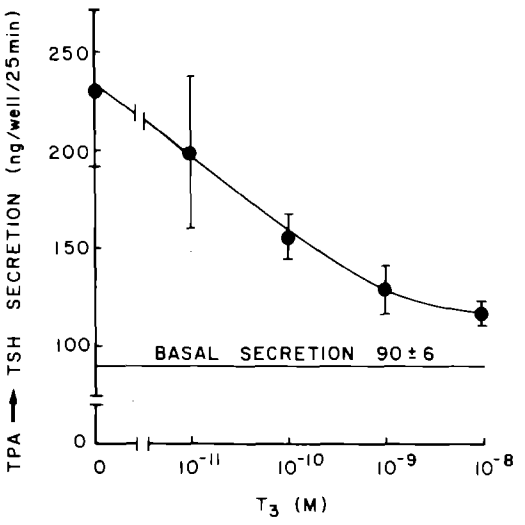

Fig. 3. Dose-response curve for T_3 inhibition of TPA-induced TSH release. Cells were incubated in the presence of various concentrations of T_3 for 170 min, with media changes at 120 and 145 min. TPA (3×10^{-7} M) was added with the last media change, and TSH secretion was measured during the ensuing 25 min. The results of 3 experiments are plotted. Basal secretion was not influenced by the T_3 concentration.

We studied whether T_3 can inhibit TPA-induced TSH secretion, as it does TRH-induced TSH secretion in this system (7). T_3 effected up to an 80% inhibition of TPA-induced TSH release, with an ED_{50} of 4×10^{-11} M (Fig. 3). The T_3 effect was significant at all doses greater than 10^{-11} M ($p<.05$, by Dunnett's test). In 4 experiments, 10^{-8} M T_3 also blocked net TSH release induced by 10^{-5} M A23187, from 102 ± 33 ng to 15 ± 15 ng ($p=.02$, by t-test).

The role of protein synthesis in the T_3 inhibition of TPA induced TSH release was studied. In 3 experiments, cycloheximide caused a mild inhibition of net 3×10^{-7} M TPA-induced TSH secretion (96 ± 9 versus 129 ± 9 ng) without inhibiting basal secretion (136 ± 12 versus 129 ± 12 ng). Net TPA-induced TSH secretion was blocked by 10^{-7} M T_3 (to 27 ± 12 ng), and this T_3 effect was prevented when cycloheximide was present with the T_3 (78 ± 18 ng; $p=.02$ versus T_3 plus TPA, by paired t-test).

Similar data were obtained in 3 experiments when the influences of cycloheximide and T_3 on TRH-induced TSH secretion

were studied. Net TSH secretion in the presence of 10^{-6} M TRH was 255 ± 63 ng, and this decreased to 108 ± 36 ng when 10^{-7} M T_3 was added. This T_3 inhibition of net TRH-induced TSH secretion was not observed when cycloheximide and T_3 were used together (252 ± 75 ng; p=.04 versus T_3 plus TRH, by paired t-test).

DISCUSSION

The binding of TRH to its cell surface receptor results in PI turnover (1,2), with formation of diacylglycerol and IP_3 (8). Activation of protein kinase C can then occur through the binding of diacylglycerol directly to this enzyme, as well as through an increase in cytosolic Ca^{++} brought about by IP_3 (8).

Phorbol esters act as diacylglycerol analogs, and thereby activate protein kinase C. That this enzyme may play a role in TRH-induced TSH secretion was suggested by the observation that TPA induces TSH secretion (9). We now show that a phorbol ester that is inactive as a tumor promoter (4α-PDD) does not stimulate TSH release, and that the TSH secretory response to a maximally effective dose of TPA is not as large as the response to TRH. This is consistent with the observation that phorbol esters bind to and activate protein kinase C in a manner similar to diacylglycerol, but that they do not elevate cytosolic Ca^{++} as does IP_3. Thus, TPA can stimulate protein kinase C activity by only 1 of the 2 mechanisms used by TRH. If protein kinase C plays a central role in TSH secretion, then the combination of TPA plus A23187 should be as potent as TRH alone. This was observed in these experiments. Furthermore, A23187 did not significantly increase the secretory response to TRH.

Although TPA and TRH were not synergistic as secretagogues of TSH, the secretion evoked by a maximally effective dose of TRH was enhanced by the addition of TPA. This would be difficult to explain if both TRH and TPA worked solely through protein kinase

C, unless it were true that TPA is a more potent activator of this enzyme than diacylglycerol in the concentration achieved under the conditions of study. The correct interpretation of these data remains to be elucidated. In addition, the observation that T_3 inhibits TPA-induced TSH release, and that this effect requires protein synthesis, suggests that T_3 inhibits both TPA and TRH-induced TSH release by a similar mechanism.

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