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In Vitro Studies on Mechanism of Action of Thyrotropin Releasing Factor* (32722)

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Recent investigations have been undertaken to discover the mechanisms whereby hypothalamic thyrotropin releasing factor (TRF) stimulates thyrotropin (TSH) secretion from the anterior pituitary. It has been shown that TRF stimulation of TSH release can be blocked competitively by thyroid hormones both *in vivo* and *in vitro* (1–3). Moreover, thyroid hormone inhibition requires intact protein synthesis, whereas TRF stimulated TSH secretion itself is not dependent upon protein synthesis (2,4,5). The recent development of a radioimmunoassay for rat TSH in this laboratory (6) has provided a sensitive and precise technique for extending studies concerning the mode of action of TRF. *In vitro* studies using this method are reported herein, and data are presented which indicate that metabolic energy is required for TRF mediated TSH release.

Materials and Methods. Bisected adenohypophyses were rapidly removed from 100-gm male Sprague-Dawley rats under a dissecting microscope and incubated singly in 5-ml Erlenmeyer flasks at 37°C in a metabolic shaker. Each flask contained 1 ml of TC-199 medium with 10% calf serum and was gassed continuously with 95% O_2–5% CO_2. For incubations with oligomycin and 2,4-dinitrophenol, Krebs–Ringer bicarbonate buffer without glucose was used to preclude substrate phosphorylation from glycolysis.

Hemipituitaries were "preincubated" for a period of 4 hours prior to experimental manipulations to remove the TSH liberated from nonviable cells. The medium was then replaced, and the glands were incubated for two successive 1-hour periods from which media was collected separately. During the first hour, no additions were made in order to determine basal TSH release. During the second hour, one hemipituitary of each pair was incubated in the presence of porcine TRF alone (kindly provided by Drs. A. Cohen and W. White of Abbott Laboratories) at a dose of 2 μg/ml medium. This TRF preparation contains 1 μU TSH/μg. The other hemipituitary was incubated with TRF plus one of the following test materials: L-thyroxine, 1 or 10 μg/ml; oligomycin, 2 μg/ml; 2,4-dinitrophenol, 10^{-4} *M*; cyclohexamide, 100 μg/ml, or ouabain, 10^{-4} *M*. Four or five hemipituitary pairs were em-

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TABLE I. Effects of TRF Alone and TRF and Varying Doses of Thyroxine on TSH Release *in Vitro*.

Treatment	Medium TSH (μU/ml) mean ± SE	Inhibition of TRF response (%)	p Value[a]
No TRF	120 ± 40	—	
TRF alone	1325 ± 55	—	
TRF + 1 μg L-thyroxine	480 ± 20	63	<.001
TRF + 10 μg L-thyroxine	165 ± 18	95	<.001

[a] For difference between response to TRF alone and TRF + thyroxine.

ployed for each experiment. Following incubation, hemipituitary pairs from several experiments were weighed on a Cahn electrobalance to 0.01 mg; no significant differences in weights were found. Media was stored at —5°C until the time of TSH assay, performed according to our recently described immunoassay method (6). Results are expressed in terms of the USP bovine TSH reference preparation which is used as a standard in this radioimmunoassay.

Results. In the absence of TRF or other substances the mean TSH concentration in the incubation medium was 140 μU per ml per hour. TRF in a dose of 2 μg/ml resulted in marked and significant increases in medium TSH concentrations, ranging in different experiments from 575–1325 μU/ml. This TRF mediated TSH release could be partially blocked by 1.0 μg/ml thyroxine added simultaneously, and was completely blocked by 10 μg/ml T_4 (Table I). In the presence of oligomycin, TRF stimulation was inhibited 55%, and 2,4-DNP at 10^{-4} M resulted in 78% inhibition (Table II). In contrast, addition of either cyclohexamide or ouabain resulted in no reduction in the TRF response. Shown in Table III are data indicating that oligomycin, 2,4-DNP, and thyroxine did not alter the basal secretion of TSH in the absence of TRF. It is inferred, therefore, that the unstimulated medium TSH is derived from cell damage and not from physiological secretory processes.

Discussion. The demonstration of TRF stimulation of TSH release *in vitro* by immunoassay provides additional support to the well-established concept of hypothalamic neurohumoral control of TSH secretion (7). The viability of the pituitary tissue under the *in vitro* conditions used was supported by the previous demonstration of net TSH synthesis and linear increase of leucine-^{14}C incorporation into protein over a 24-hour period of incubation (5).

The L-thyroxine was capable of almost completely preventing TRF stimulation in

TABLE II. Effects of Various Metabolic Inhibitors on TRF Mediated TSH Release.

Treatment	Medium TSH (μU/ml) mean ± SE	Inhibition of TRF response (%)	p Value[a]
No TRF	140 ± 25	—	
TRF alone	1226 ± 70	—	
TRF + oligomycin	626 ± 25	55	<.001
TRF alone	860 ± 25	—	
TRF + 2,4-DNP	210 ± 15	78	<.001
TRF alone	990 ± 35	—	
TRF + cyclohexamide	950 ± 45	10	>.05
TRF alone	575 ± 25	—	
TRF + ouabain	625 ± 30	0	>.05

[a] For difference between response to TRF alone and TRF and inhibitor.

TABLE III. Effects of Inhibitors and L-Thyroxine upon Pituitary TSH Secretion in the Absence of TRF.

Treatment	Medium TSH (μU/ml) mean ± SE	p Values
Medium alone	141 ± 18	>0.5
Medium + 100 μg L-thyroxine	105 ± 13	
Medium alone	245 ± 14	>0.5
Medium + 2,4-DNP	293 ± 22	
Medium alone	123 ± 16	>0.5
Medium + oligomycin	145 ± 20	

this *in vitro* system, corroborating the previous data of Vale and co-workers (3), in which thyroxine inhibition at the pituitary level was shown. It should be pointed out, however, that the absolute free thyroxine concentration *in vitro* necessary to produce this effect was probably greater than *in vivo* free thyroxine concentrations by a factor of more than 10^5. Moreover, the physiological contribution of this direct pituitary negative feedback *in vivo* in the overall scheme of hypothalamic pituitary thyroid interactions remains unsettled, since there is only limited knowledge at present regarding thyroxine influences upon TRF synthesis and secretion (8).

Significant inhibition of TRF activated secretion was observed in the presence of both oligomycin and 2,4-dinitrophenol. Both of these compounds interfere with mitochondrial generation of high energy intermediates. However, the postulated locus of action of dinitrophenol in the mitochondrial coupling sequence is earlier than that of oligomycin. Dinitrophenol hydrolyzes an early high energy compound, and stimulates mitochondrial adenosine triphosphatase activity (9), whereas oligomycin is thought to combine with the last coupling intermediate so that inorganic phosphate cannot be utilized to form ATP. Thus, one may infer that mitochondrial ATP, or an earlier high energy intermediate, is required for TRF action.

How metabolic energy is involved in TSH secretion is currently under study. If a membrane adenosine triphosphatase in the pituitary cell plasma membrane is linked to TSH transport from the cell, it is distinct qualitatively from the class of adenosine triphosphatase systems in red blood cells and other tissues that are inhibited by ouabain (10). The failure of cyclohexamide, an inhibitor of protein synthesis, to block TRF action implies that secreted TSH presumably derives from an intrapituitary pool of stored TSH instead of from more recently synthesized TSH.

Summary. The TSH secretion from hemipituitary glands is stimulated *in vitro* by TRF and this action is blocked by large doses of L-thyroxine. The response to TRF is inhibited by both 2,4-dinitrophenol and oligomycin, indicating that energy is required for TRF action. It is not inhibited by cyclohexamide or ouabain.

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