ABSENCE OF BINDING OF THYROID-STIMULATING HORMONE SUBUNITS TO SPECIFIC SITES OF THYROID CELLS

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1. Introduction

The binding of ^{125}I-labeled thyriod-stimulating hormone (TSH) to specific high affinity sites of porcine thyroid cells reassociated in culture into follicles and to their derived plasma membranes has been recently demonstrated [1,2]. Thyrotropin is a glycoprotein formed of two chemically dissimilar subunits. The α-subunit is identical to the α-subunit of other glycoprotein hormones (LH, FSH, hCG) whereas the β-subunit is hormone specific [3]. The part of the TSH molecule which binds to the receptor site of the thyroid cell is still unknown. The individual subunits are of very low biological activity in vivo suggesting that they do not bind to the TSH-specific receptor sites. However, it is possible that one of the subunits or both could bind to the sites without eliciting the hormonal response.

In this letter, we show that TSH subunits neither bind to intact thyroid cells nor compete with the bining of ^{125}I-labelled TSH.

2. Materials and methods

Isolated porcine thyroid cells were obtained as previously described [4] and seeded at a concentration of 3×10^6 cells per ml of Eagle minimum essential medium pH 7.4 containing 20% calf serum, penicillin (200 U/ml); streptomycin sulfate (50 μg/ml) (medium E), and 0.2 mM dibutyryl cyclic AMP in 75 cm^2 Falcon plastic bottles and incubated at 35°C in 95% air–5% CO_2. After 4 or 5 days incubation the cells were collected by EGTA treatment and gentle scraping and immediately used for binding experiments which were carried out at equilibrium in exactly the same conditions as described previously [1–2]. KB cells were cultured in the same medium up to confluence and were detached from the support by the same procudure. Porcine TSH and its α- and β-subunits where initially prepared by the countercurrent distribution method (TSH-SSD) of Liao et al. [5] and the propionic acid method (TSH-α, -β, PA) of Liao and Pierce [6]. A second set of subunit preparations was purified by chromatography according to Closset and Hennen [7] and will be referred to as TSH-α, -β, Chrom. Porcine LH and its subunits were prepared as described by Hennen et al. [8].

Enzymatic labelling with ^{125}I of pTSH and its subunits was performed according to Jaquet et al. [9] using lactoperoxidase and hydrogen peroxide as the oxidizing agent. The labeled hormone or its subunits contained 1.5 to 2.0 iodine atoms/mole with a specific radioactivity of 1.7 to 2.5 Ci/μmole. The TSH content of the α-subunit was assessed by radioimmunoassay. [^{125}I]TSH was used as tracer. The antiserum was selected for its specificity to the antigenic determinants

of the β-subunit and was found to be devoid of antibody against the α-subunit. As the β-subunit cross-reacted extensively in this system, no figure for TSH contamination of this subunit could be assumed.

The thyrostimulating activity of hormonal preparations was determined by the McKenzie bioassay in mice [10] and by a new in vitro test based on the property of TSH to stimulate the reorganization into follicles of thyroid cells. Briefly, 15×10^6 cells are seeded in 25 cm^2 plastic Falcon bottles and serial dilutions of hormonal preparations are added to the culture medium. The bottles are observed each day for the presence of cells reassociated into follicles. The minimum amount of TSH inducing a positive response at day 3 of culture is noted and compared to the minimum amount of serial dilutions of LH, TSH and LH subunit preparations giving a positive response. Eight serial dilutions of each compound in duplicate were routinely performed.

3. Results

Table 1 shows the thyrostimulating activity of the preparations of porcine TSH, LH and their subunits used in these studies as determined by the in vivo assay in mice and the in vitro bioassay based on the ability of TSH to stimulate the histiotypic reorganization into follicles of isolated thyroid cells. Both methods showed that the TSH β-subunit prepared by two different procedures (PA and Chrom) possess no or negligible TSH activity. The TSH-α PA-preparation exhibited 2.5 and 10% of the native hormone activity by the in vivo and the in vitro methods, respectively. However, the TSH-α Chrom-preparation showed a very low TSH activity (0.07%) as shown by the in vivo assay. The evaluation of TSH contamination of the TSH-α preparations by radioimmunoassay gave figures which paralleled the activity of those reagents as measured in the in vivo and in vitro bioassays. No or negligible TSH activities were detected in porcine LH and its subunits indicating absence of contamination by native TSH.

The binding of ^{125}I-labeled TSH, its subunits and LH-α was studied using cultured thyroid cells reorganized into follicles under the influence of dibutyryl cyclic AMP. KB cells served as controls. As shown in table 2 none of the subunits specifically bound to both cell types. All the values of bound radioactivity were below those of [^{125}I]TSH non-specific binding to thyroid cells. These results indicate that TSH subunits and LH-α do not bind to the specific TSH receptor sites of thyroid cells. They were confirmed by competition binding experiments. At a concentration of

Table 1
Thyrostimulating activity of preparations of porcine TSH, porcine LH and their subunits

Compounds tested	McKenzie's bioassay	Histiotypic reassociation of thyroid cells into follicles*	Radioimmunoassay
	IU/mg	μg/ml	% TSH contamination
pTSH	37	0.002	
pTSH-α (PA)	0.9 (2.4)	0.02 (10)	10.0
pTSH-α (Chrom)	0.024 (0.07)	nd	2.0
pTSH-β (PA)	<0.01**	2.5 (1×10^{-3})	
pTSH-β (Chrom)	<0.01	nd	
pLH	≤0.01	0.4 (2.5×10^{-3})	<0.1
pLH-α	<0.01	5.75 (3×10^{-4})	<0.1
pLH-β	<0.01	57.5 (3×10^{-5})	<0.1

* minimum amount of hormone inducing a positive response.
** means that no response was observed on the basis of the given figures nd, not determined. In parenthesis, calculated % of TSH that would contaminate the preparations if they do not possess intrinsic thyrostimulating activity.

Table 2
Binding of radioiodinated pTSH, pTSH-α, pTSH-β and pLH-α to dibutyryl cyclic AMP-stimulated thyroid cells and to KB cells.

Radioiodinated hormone or subunit	Thyroid cells	KB cells
	cpm/2 × 10^6 cells	
pTSH*	3128 ± 85	220 ± 30
pTSH* + unlabeled		
pTSH (25 μg)	610 ± 125	235 ± 25
pTSH-α*	219 ± 40	–
pTSH-β	329 ± 50	264 ± 52
pLH-α	216 ± 72	73 ± 21
pLH-α* + pTSH-β*	550 ± 138	245 ± 104

* ^{125}I-labelled preparations. Assays performed with thyroid cells cultured for 5 days in the presence of 0.2 mM dibutyryl cyclic AMP or with KB cells at confluence. Cells (2 × 10^6) were incubated in 0.5 ml HEPES-medium E at 35°C for 20 min in the presence of 0.39 nM radioiodinated hormone or subunits. Adsorption on tube walls substracted. Assays in triplicate. Addition of an excess (1.7 μM) of unlabeled subunits does not modify the amount of radioactivity bound to cells. pTSH-α and pTSH-β Chrom-preparations were used throughout.

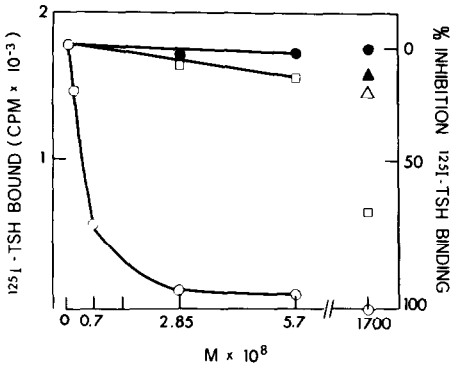

Fig. 1. Effect of pTSH, its subunits and pLH subunits on ^{125}I-labeled pTSH binding to thyroid cells. Thyroid cells (3 × 10^6) cultured for 4 days in the presence of 0.4 mM dibutyryl cyclic AMP were incubated for 20 min with [^{125}I] TSH (0.35 nM) and increasing concentrations of unlabeled hormone or subunits to obtain the final concentration plotted in abcissa. Additions: pTSH (○—○—○); pTSH-α (□—□—□); pTSH-β (●—●—●); pLH-α (△); pLH-β (▲). Each experimental point is the mean of closely agreeing triplicates. Two experiments were performed with similar results.

57 nM, native unlabelled TSH almost completely (95%) inhibits the binding of [^{125}I]TSH to thyroid cells. At concentrations up to 1.7 μM, TSH-β is ineffective whereas unlabelled TSH-α (57 nM) inhibits ^{125}I-labelled TSH binding by about 10%. Calculated in term of contamination with native TSH this value corresponds to 0.04% which is in very good agreement with that found using the bioassay (0.07%) (table 1). LH-α and -β subunits at 1.7 μM inhibit ^{125}I-labeled TSH binding by no more than 15%, indicating very small contamination by TSH.

4. Discussion and conclusions

The availability of purified subunit preparations of glycoprotein hormones has rendered possible both in vivo and in vitro hormone activity measurements of the subunits and their binding to receptor sites in target tissues [11–13]. Such studies critically depend on the purity of the subunit preparations and it is sometimes difficult to relate small amounts of biological activity in certain preparations to either true intrinsic activity or contamination by the native hormone. Therefore, the interpretation of binding experiments using glycoprotein hormone subunits should depend on the available criteria of purity of the subunit preparations used.

In the present studies, our conclusion that TSH subunits do not bind to the specific TSH receptor sites of thyroid cells was assessed from the following considerations: 1) the subunits of pTSH prepared by the new chromatographic method showed a high degree of chemical purity which allowed the determination of their primary sequence [7]; 2) TSH-β (Chrom) showed no thyrostimulating activity using both in vivo (mouse) and in vitro (reassociation of isolated thyroid cells into follicles) bioassays. TSH-α (Chrom) possessed a very small activity in vivo but a relatively high level of presumed contamination with native TSH as shown by radioimmunoassay; since the latter is based upon β-subunits, this result may well be related to contamination with small amounts of free β-subunit in itself biologically inactive; 3) As compared to KB cells, dibutyryl cyclic AMP-induced reassociated thyroid cells which show a full complement of TSH receptor sites [1] were not able to bind ^{125}I-labeled TSH-α or

TSH-β; 4) unlabeled TSH-β could not compete with the binding of [^{125}I]TSH to thyroid cells whereas at a concentration of unlabeled TSH which inhibits 95% of [^{125}I]TSH binding, TSH-α provoked about 10% inhibition. This is likely due to a small contamination by native TSH since on this basis, a value of 0.04% contamination could be calculated which agreed fairly well with the value of 0.07% derived from the bioassay in mice. In addition, pLH-α which is structurally identical to pTSH-α did not substantially inhibit [^{125}I] TSH binding at 30-times the concentration of unlabeled TSH which inhibited 95% of labeled-TSH binding.

It is therefore concluded that binding to specific receptor sites of the thyroid cell and further stimulation of physiological effects require a TSH subunit conformation such as that realized in the native hormone. A recent study on the properties of the subunits of human chorionic gonadotropin concluded that any detectable biological activity in the subunit preparations likely resulted of small contamination with the native hormone and not of intrinsic activity of the subunit molecules themselves [14].

Our results disagree with those reported by Amir et al. [15] who found that both α- and β- subunits of bovine TSH bound to bovine plasma membranes whereas only the β-subunit inhibited tritiated-TSH binding. Comparison of the results is made difficult due to different methodologies e.g. low specific radioactivity of the tritiated TSH or its subunits used by Amir et al. (about 1:100 that obtained for [^{125}I]TSH in our studies) and the absence of control of possible contamination of the subunit preparations by native TSH.

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