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Research Article

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Unlike Thyrotropin, Thyroid-stimulating Antibodies do not Activate Phospholipase C in Human Thyroid Slices

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Abstract

The effects of thyroid-stimulating antibodies (TSAb) and of thyrotropin (TSH) were compared, on the generation of cyclic AMP and inositol phosphates (InsP), in human thyroid slices incubated in vitro, and on the Rapoport cyclic AMP bioassay. The TSAb positive sera were obtained from 19 patients with Graves' disease. In 14 experiments with the slices system, TSH significantly increased cyclic AMP accumulation (TSH, 0.03–10 mU/ml) as well as the cyclic AMP-independent inositol trisphosphate ($InsP_3$) generation (TSH, 1–10 mU/ml). In the same 14 experiments, TSAb (0.10–28 mg/ml) enhanced cyclic AMP intracellular levels as expected while they did not induce any InsP accumulation. Even when TSAb increased cyclic AMP levels to the same or higher values as those obtained with TSH concentrations allowing $InsP_3$ generation, TSAb were still unable to activate the phosphatidylinositol-Ca^{2+} cascade. The patterns of the response curves of TSAb and TSH on cyclic AMP accumulation were different, suggesting that different mechanisms may be involved. In addition, unlike TSH, TSAb were not able to stimulate H_2O_2 generation, which in human tissue mainly depends on the activation of the phosphatidylinositol-Ca^{2+} cascade. Immunoglobulins from six additional Graves' patients lacking measurable cyclic AMP-stimulating activity in both slices and cells systems did not activate phospholipase C either. In conclusion, our results show that TSAb do not share all the metabolic actions of TSH on human thyroid tissue. The data provide support for the concept that the pathogenesis of Graves' disease can be fully accounted for by the ability of TSAb to stimulate adenylate cyclase. This work also confirms that TSH activates the cyclic AMP and the phosphatidylinositol cascade by independent pathways in the human thyroid. (*J. Clin. Invest.* 1991. 87:1634–1642.) Key words: human thyroid • thyroid-stimulating antibodies • thyrotropin • inositol triphosphate • cyclic AMP

Introduction

Thyroid hyperfunction and growth in Graves' disease is ascribed to the thyrotropin (TSH)[1]-mimicking effect of autoantibodies directed against the TSH receptor on the surface of thyroid cells (for a review see references 1 and 2). In human thyroid, we have previously shown that TSH could activate the two main pathways of cell regulation (3): the cyclic AMP cascade in which activation of adenylate cyclase generates cyclic AMP, and the phosphatidylinositol cascade in which the receptor-activated phospholipase C releases two second messengers from phosphatidylinositol 4,5-bisphosphate: diacylglycerol and inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$)-$Ins(1,4,5)P_3$ itself activating Ca^{2+} release from intracellular stores (4). We have shown that $InsP_3$ accumulation was cAMP-independent (3). Several methods are currently used for the detection of TSAb, mainly based on adenylate cyclase stimulation, as measured in human thyroid slices, cells or membranes, or in FRTL5 cells (2). Enhancement of cyclic AMP accumulation could certainly account for the known effect of TSAb on the human thyroid. Indeed, in dog and human thyroid, hormone secretion is stimulated by agents that increase cyclic AMP levels and inhibited by those that increase Ca^{2+} levels or activate protein kinase C (5–7). Cyclic AMP mediates the TSH-induced thyroid cell differentiation and proliferation (8, 9). However, claims have appeared for the existence of a growth-stimulating immunoglobulin (thyroid growth-stimulating antibodies or TGSAb), separate from TSAb, that might cause thyroid growth in Graves' disease and some euthyroid goiters through a pathway distinct from adenylate cyclase (10–13). These results were in contradiction with the findings of Zakarija et al. who showed a strict parallelism between growth and cyclic AMP accumulation-stimulating activities in Graves' immunoglobulins (14). These in vitro studies on human thyroid slices were initiated to determine whether Graves' disease immunoglobulins reproduced one or both of the primary metabolic effects of TSH (adenylate cyclase and phospholipase C activation) that might be relevant to the increased function of the gland and essential for the development of thyrotoxicosis.

Methods

TSAb. Sera were obtained from 25 patients with hyperthyroidism due to Graves' disease (20 women, 5 men; age 17–58, mean 53). The diagnosis was based on the classical clinical findings of thyrotoxicosis (with or without goiter or ophthalmopathy) associated with elevated T4, T3, and undetectable TSH levels, and a diffuse radioactive iodine uptake. Blood samples were obtained at the time when diagnosis was made in 16 patients while 9 of them were under methimazole therapy. The number of sera without measurable TSAb activity in both the Rapoport and the slices' assay systems was not different in the group of treated patients (4 of 16, 25%) than in the untreated group (2 of 9, 22%). Serum was separated by centrifugation of 50 ml clotted blood. Control sera of healthy subjects were also used. Crude IgG was prepared by ammonium sulfate precipitation (15). After precipitation and centrifugation, water was added to the pellet until complete dissolution and the IgG concentration was measured by its extinction coefficient at 280 nM. Samples were then dialyzed against NaCl free Hanks' medium for experiments on cells and against Krebs-Ringer bicarbonate (KRB)

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1. *Abbreviations used in this paper:* ASP, ammonium sulfate precipitates; InsP, inositol phosphates; KRB, Krebs-Ringer bicarbonate buffer; TSAb, thyroid-stimulating antibodies; TSH, thyrotropin.

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buffer for experiments on slices. Preparation of highly purified IgG on protein A sepharose columns was performed for two samples and provided no advantage in comparison with the ammonium sulfate precipitation technique. TSAb activities in the IgG fractions were checked in the Rapoport bioassay (15). Briefly, monolayer cultures of human thyrocytes, which had been frozen in liquid N2 after primary culture and then thawed, were exposed to TSH or TSAb in hypotonic medium (NaCl free Hanks) for 4 h at 37°C. Each assay contained TSH standards diluted in control IgG used at the same concentration as the TSAb samples. Cyclic AMP released into the medium was measured by RIA (15).

Human thyroid tissue. For slices experiments, human thyroid tissue was obtained from euthyroid patients undergoing lobectomies for resection of solitary "cold" nodules. Only the healthy normal looking nonnodular tissue was used and cut into thin slices of ~ 50 mg wet wt with a Stadie-Riggs microtome within 10–30 min of surgical resection. Slices were randomly assigned to inositol phosphates and cyclic AMP experiments. For cells in culture, thyroid tissue came from surgically treated Graves' patients.

Inositol phosphates measurements. Slices were incubated at 37°C, under an atmosphere of O_2/CO_2 (15:5, vol/vol) in 2 ml of KRB, supplemented with 8 mM glucose, and 0.5 g/liter of bovine serum albumin. In the first 4-h preincubation, 20 μCi/ml ^{3}H-inositol (sp act 16.5 Ci/mmol) was added to this medium. Tissue was then transferred to fresh unlabeled incubation medium to which lithium was added after 15 min. After a further 5 min, slices were put for 1 h in a third series of flasks containing the tested agents (TSH, TSAb, control IgG) in the presence of 10 mM LiCl. Incubation was stopped by rapid immersion of the slices in (3%) ice-cold perchloric acid. After homogenization and centrifugation (2,000-*g*/10 min), the pellet was washed with 1 ml of 1% cold perchloric acid and recentrifuged. The combined supernatants were neutralized to pH 7.8 by the addition of 760 mM KOH in the presence of a 380-mM Hepes buffer. ^{3}H-labeled inositol phosphates from the supernatants were eluted in a stepwise fashion through an anion exchange column of AG_1-X_8 resin (formate form, 100–200 mesh; Biorad, Watford, UK) (16). Intracellular $InsP_3$ is expressed as cpm/100 mg wet wt tissue. Incorporation of ^{3}H-inositol into the total phosphatidylinositides pool was estimated after chloroform/methanol extraction of lipids from the pellet (17).

Cyclic AMP measurements. Slices were incubated following the same protocol as for inositol phosphates measurements but without ^{3}H-inositol. For the test incubation of 1 h, medium was supplemented with 100 μM Ro 20-1724 as a cyclic AMP-specific phosphodiesterase inhibitor. The slices were then dropped into boiling water for 5 min, homogenized, centrifuged, and the supernatant lyophilized. The tissue extracts were resuspended in water and cyclic AMP was measured in a binding assay using protein kinase from bovine skeletal muscle (18). Intracellular cyclic AMP concentrations were expressed as pmol/100 mg wet wt tissue.

H_2O_2 determinations. Measurements were performed according to the method of Benard and Brault (19) based on the conversion of the nonfluorescent substrate homovanillic acid to a fluorescent derivative in the presence of H_2O_2 and peroxidase. Slices were preincubated for 1 h in KRB buffer supplemented with 8 mM glucose and 0.5 g/liter BSA, then transferred to fresh medium containing 0.1 mg/ml horse radish peroxidase type II, 440 μM homovanillic acid and the agonist tested. The fluorescence of the medium was measured 90 min later (20).

Materials. TSH was used as Thytropar (4 IU/mg) (Armour Pharmaceutical Co., Phoenix, AZ). Myo-2 ^{3}H-inositol came from New England Nuclear (DuPont-NEN, Haren, Belgium). Ro 20-1724 was a gift from Hoffmann-La Roche (Nutley, NY). All other reagents were of the purest grade commercially available. Purified bovine TSH (40 IU/mg) was a gift from Dr. J. G. Pierce (UCLA, Center for Health Science, Los Angeles, California) and purified human TSH (11 IU/mg) came from Porton Products (Salisbury, Great Britain).

Statistical analysis. For the comparison of agonist and control groups the Student's *t* test was used. Each experimental condition was conducted with triplicate flasks and slices for $InsP_3$ and H_2O_2 measurements and with duplicates for cyclic AMP determinations. $P < 0.05$ was chosen as the level of significance.

Results

Effects of Graves' immunoglobulins on cyclic AMP accumulation. Among the 25 ammonium sulfate precipitates (ASP) tested, 6 were negative for cyclic AMP-stimulating activity in both the Rapoport and the slices system, while 19 of them were active in one or both of the assays. In the Rapoport bioassay (15), the potency of the TSAb were expressed as equivalents of mU TSH in the assay and ranged from 0.001–10 mU TSH (Fig. 1). All ASP were then used for cyclic AMP determinations on human thyroid slices, in which their activities were also expressed as equivalents of mU TSH (range 0–3.3 mU TSH). As expected from the lower sensitivity of the slices system, three sera showed cyclic AMP activities in the first assay without any activity in the second (No. 17–19). One ASP (No. 14) had no detectable cyclic AMP-generating activity in the Rapoport bioassay, but it was positive in the slices system. For the 15 other ASP, there was a good correlation between the activities in both assays ($r = 0.71$, $n = 15$, $P < .01$). The activity of TSAb preparations was largely independent of the origin of the human thyroid tissue used for the test. Indeed, 7 ASP used at constant concentrations had the same relative activity versus a standard TSH concentration action curve in two or three different thyroid tissues (data not shown). As observed earlier, there was no correlation between the extent of cyclic AMP enhancement and clinical parameters, such as goiter size, severity of hyperthyroidism, and/or ophthalmopathy (21).

Comparison of cyclic AMP and $InsP_3$ generations induced by TSH and TSAb. 14 experiments using ASP having the strongest TSAb activities (nb 1 to 14 on Fig. 1) were performed on 14 different thyroid tissues. The results of the individual experiments are shown in Fig. 2. In all the experiments, TSH (0.03–

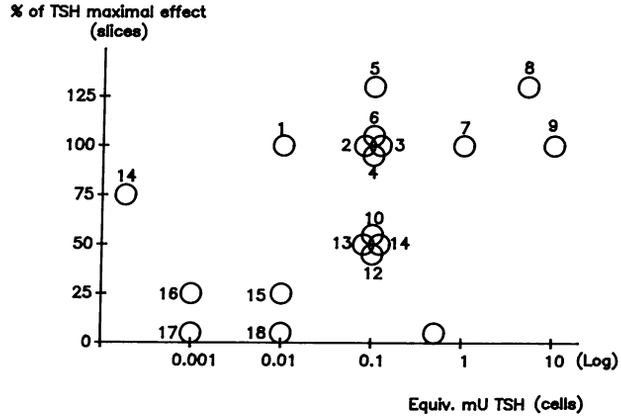


Figure 1. Comparison of the strength of cyclic AMP-stimulating activities for Graves' sera No. 1–19 in the Rapoport bioassay (abscissa) and in our in vitro incubated slices system (ordinate). TSAb were used for maximal concentrations ranging from 0.1 to 28 mg/ml (as expressed in Fig. 2). Their potencies are expressed as equivalents of mU TSH (*Equiv. mU TSH*) and ranged from 0 to 10 for cells. For slices, some TSAb cyclic AMP-stimulating activities (5, 8) exceeded highest cyclic AMP stimulation levels obtained with TSH. Correlation coefficient, $r = 0,71$; $P < 0.01$.

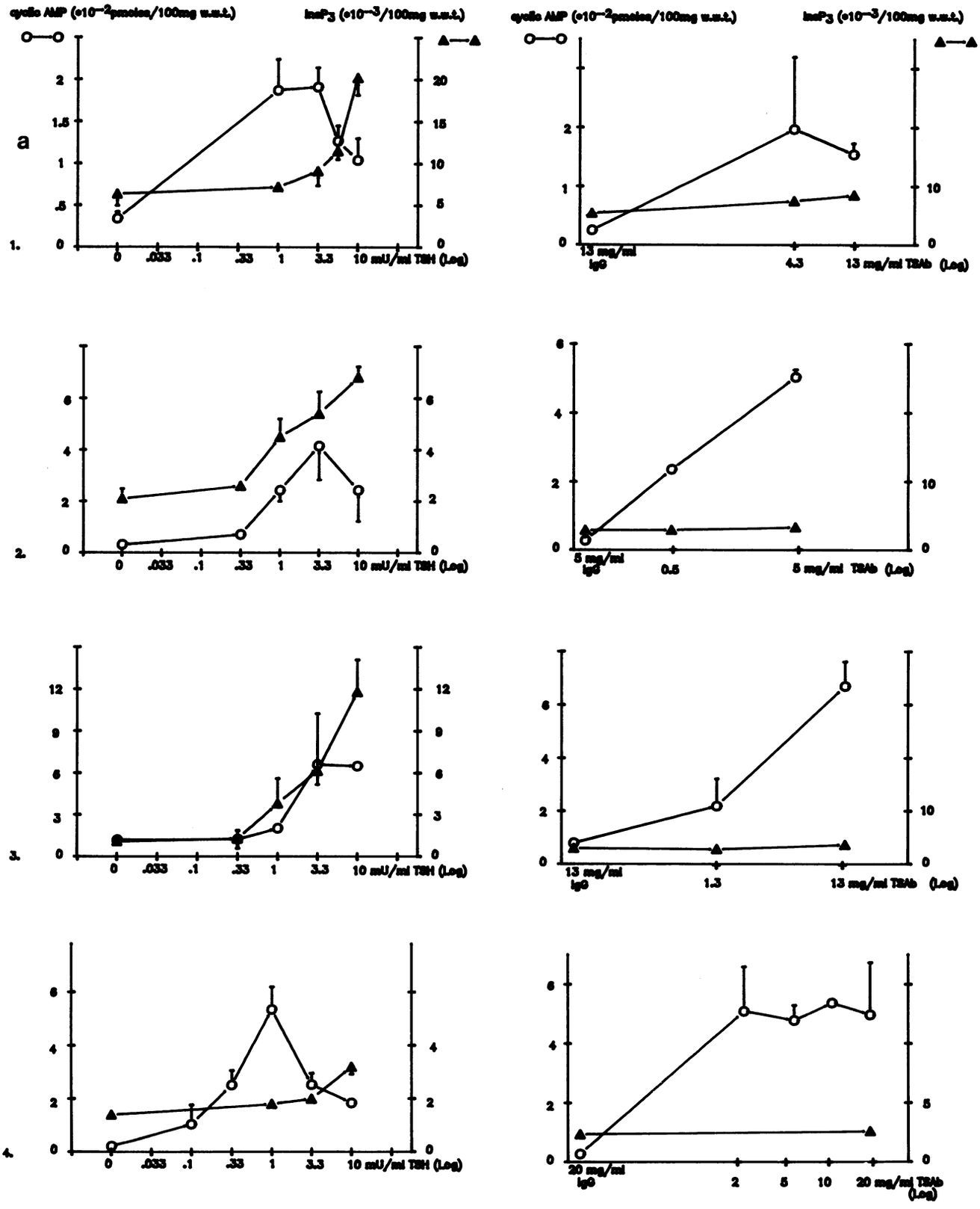
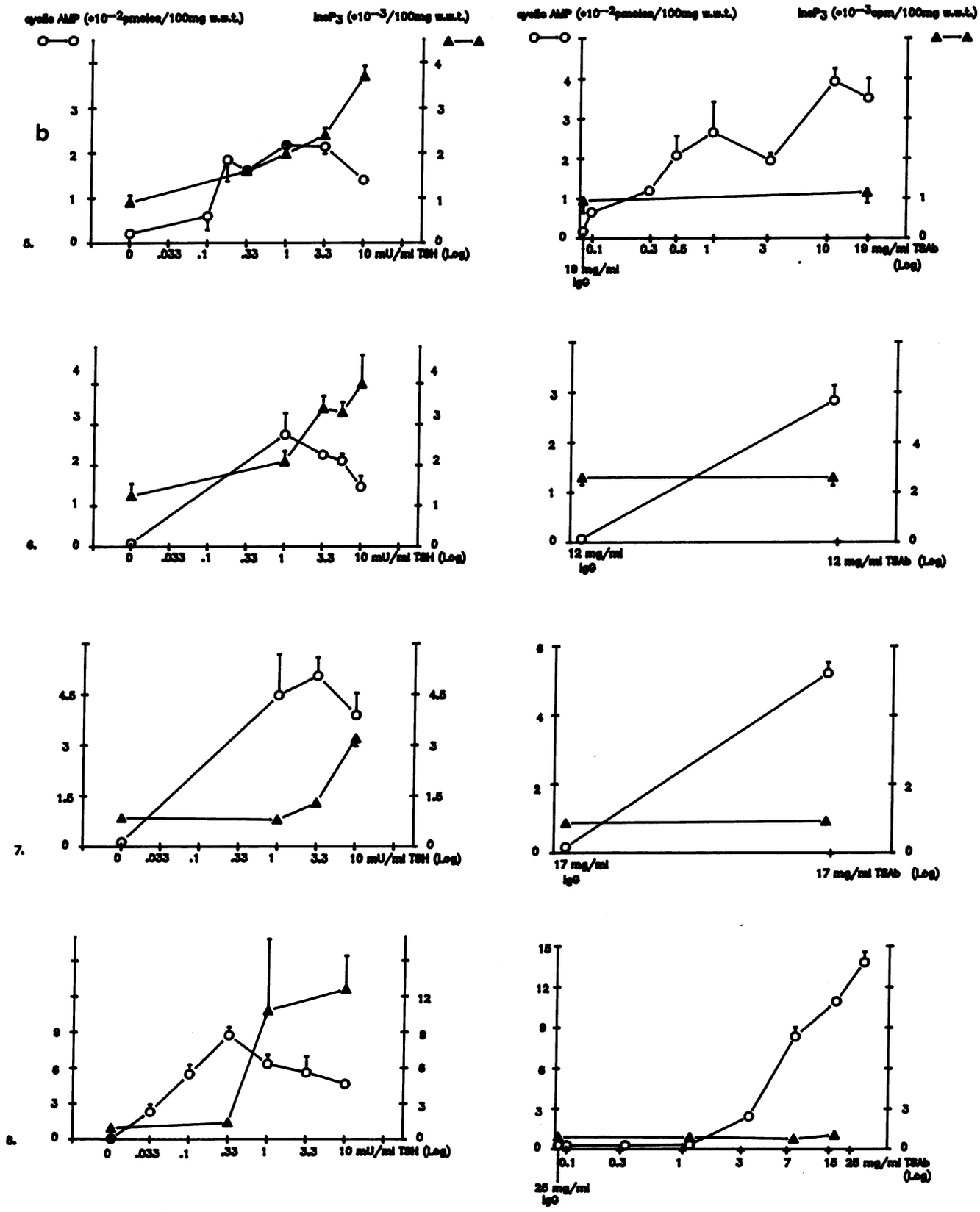


Figure 2. Individual results of the 14 experiments using TSAb sera No. 1–14. The number of the experiment corresponds to the number of the sera expressed in Fig. 1. (— o —) Cyclic AMP concentrations-action curves in response to TSH or TSAb. (— ▲ —) $InsP_3$ concentrations-action curves in response to TSH or TSAb. The first point on the abscissae for the TSAb panel refers to normal sera containing nonspecific immunoglobulins at a concentration equivalent to the highest TSAb concentration used in the experiment. w.w.t., wet weight tissue. Results are expressed as means±SEM of triplicates for $InsP_3$ determinations and ±SD of duplicates for cyclic AMP measurements. SEM and SD are not drawed when inferior to the size of circles and triangle symbols.



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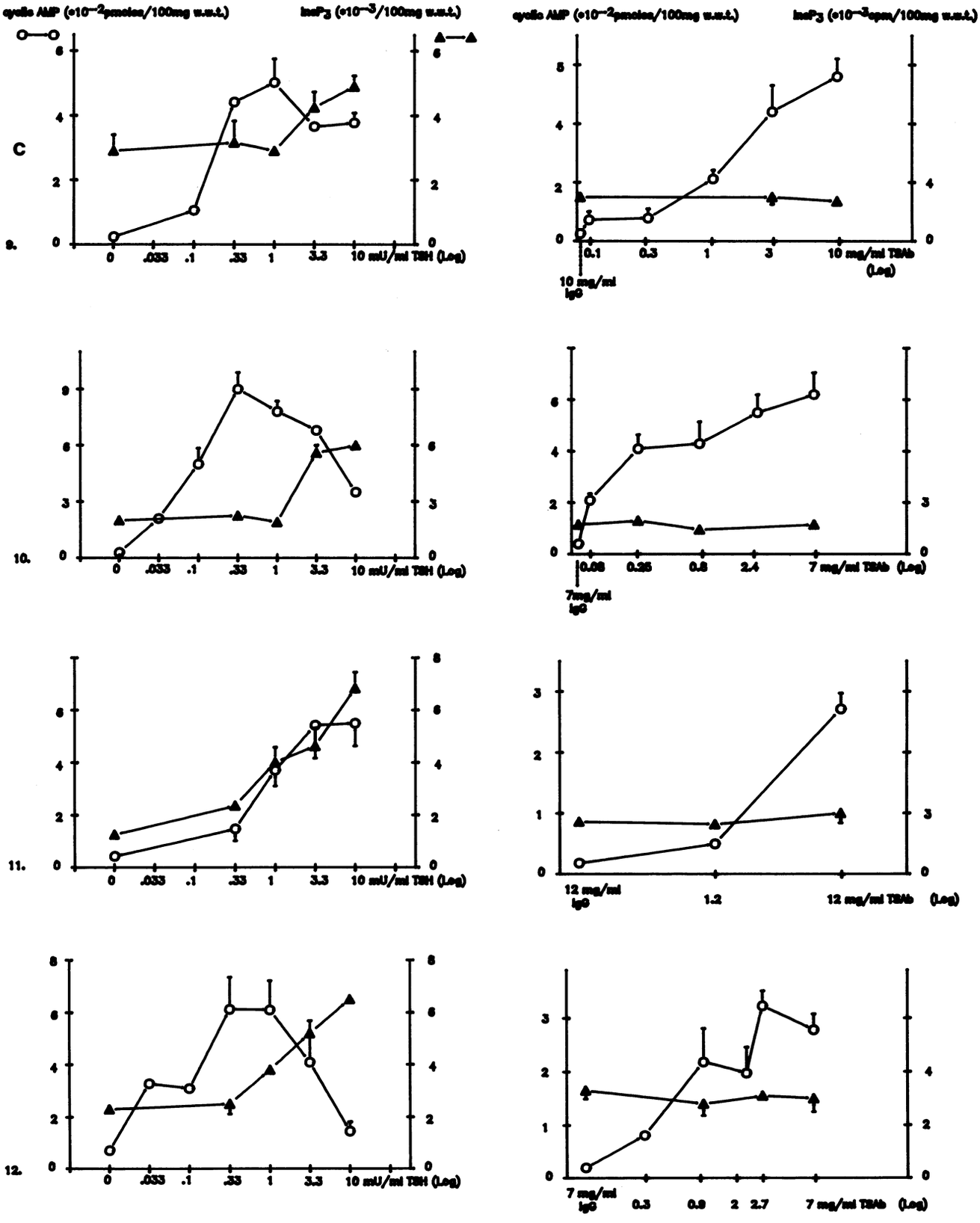

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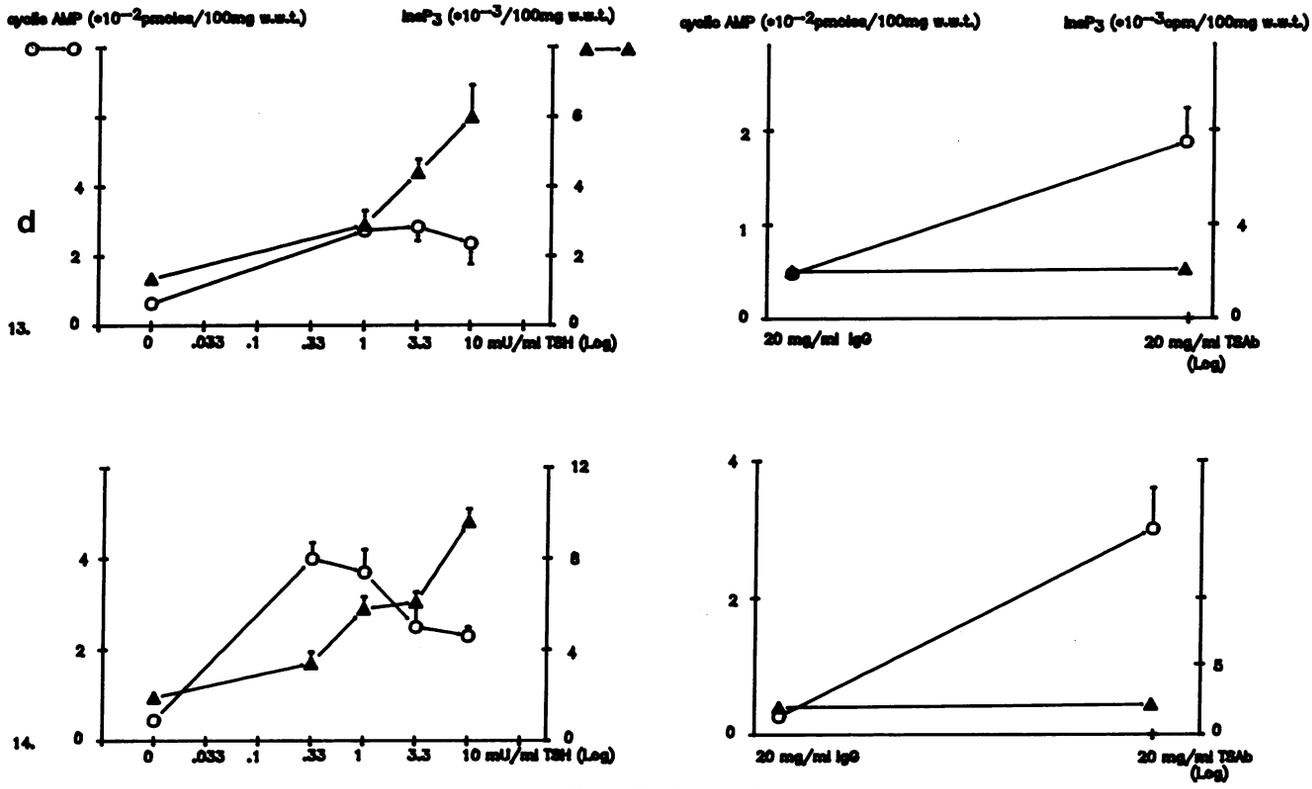

Figure 2. (Continued)

10 mU/ml) significantly increased cyclic AMP accumulation and $InsP_3$ generation, the latter at higher concentrations (TSH, 1–10 mU/ml). In the same experiments, TSAb (0.10–28 mg/ml) also enhanced cyclic AMP levels but did not induce $InsP_3$ accumulation. Dilutions of Graves' patients IgG did not reveal any $InsP_3$ effect. When analysis of the data was restricted to the particular subset of TSAb, which increased cyclic AMP to the same values as those obtained using TSH concentrations allowing $InsP_3$ generation (experiments 1–10, n = 10), no TSAb effect on the latter became evident. The same results were found for the inositol monophosphate and inositol bisphosphate fractions, while the total ^{3}H-inositol content of the phospholipid pellet was not influenced by TSH or TSAb (data not shown).

In 7 experiments (2, 3, 4, 6, 11, 12, 13), control IgG from healthy subjects induced a slight increase in $InsP_3$ generation of the same magnitude as the TSAb in the same experiments. This effect was thus related to the thyroid sample studied and not to any differential characteristics of the normal and Graves' IgG. The lack of effect of TSAb on inositol phosphates generation was not related to a negative interaction of Graves' IgG with receptor activated phospholipase C, since TSH-induced $InsP_3$ responses were not inhibited by Graves' sera (3 experiments using sera 9, 11, 14). Fig. 3 shows the results for one of these experiments (serum No. 11).

Cases 4, 5, 8, 9, 10, and 12 in Fig. 2 show more detailed TSAb and TSH concentration-response curves for cyclic AMP and $InsP_3$ accumulations. In these experiments, the TSH-induced cyclic AMP generation curve was biphasic (i.e., decreasing cyclic AMP for the highest TSH concentrations), while that for TSAb showed a linear concentration dependence or a plateau phase.

Effects of TSAb in comparison with TSH on H_2O_2 generation. 8 of the most potent TSAb ASP (No. 1–8) were tested on slices in order to determine whether they could induce H_2O_2 production (Table I). H_2O_2 values for TSAb-stimulated slices were not different from the control values, except for TSAb No. 6 which inhibited H_2O_2 generation. TSH 10 mU/ml induced a 12-fold increase of H_2O_2 generation.

Comparison of cAMP and $InsP_3$ generations induced by thytropar, bovine or human purified TSH preparations. Fig. 4 shows that the biphasic effect of TSH on cAMP is produced by the commercial and purified bovine TSH preparation, as well as by purified human TSH.

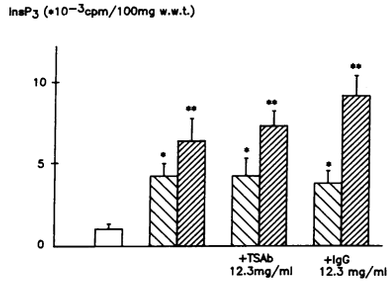

Figure 3. Lack of effect of Graves' IgG (*TSAb*) or healthy subjects' IgG (*IgG*) on TSH 3 and 10 mU/ml-induced $InsP_3$ accumulations. Serum used is number 11 on Fig. 1. w.w.t., wet weight tissue. Results are expressed as means±SEM. $^*P < 0.05$; $^{**}P < 0.01$.

Table I. Effect of nonspecific immunoglobulins from normal subjects (IgG), TSAb (1–8), and TSH 10 mU/ml on H_2O_2 generation in human thyroid slices

Agent		Concentration	H_2O_2 ng/100 mg WWT × 60 min
	n	*mg/ml*	
BSA	(3)	19	69±39
IgG	(3)	15	60±28
TSAb 1	(2)	13	57±27
2	(2)	4.6	75±24
3	(3)	10.9	37±16
4	(3)	20	42±10
5	(2)	19	41±16
6	(2)	12	20±15*
7	(2)	20	69±42
8	(2)	25	72±37
TSH	(3)	10 mU/ml	847±106‡

Numbers ($TSAb_1$ to $TSAb_8$) refer to the Graves' sera presented in Fig. 1. W.W.T, wet weight tissue. Results are expressed as means±SD of triplicates. * $P < 0.05$, ‡ $P < 0.01$; *n*, number of experiments in triplicates.

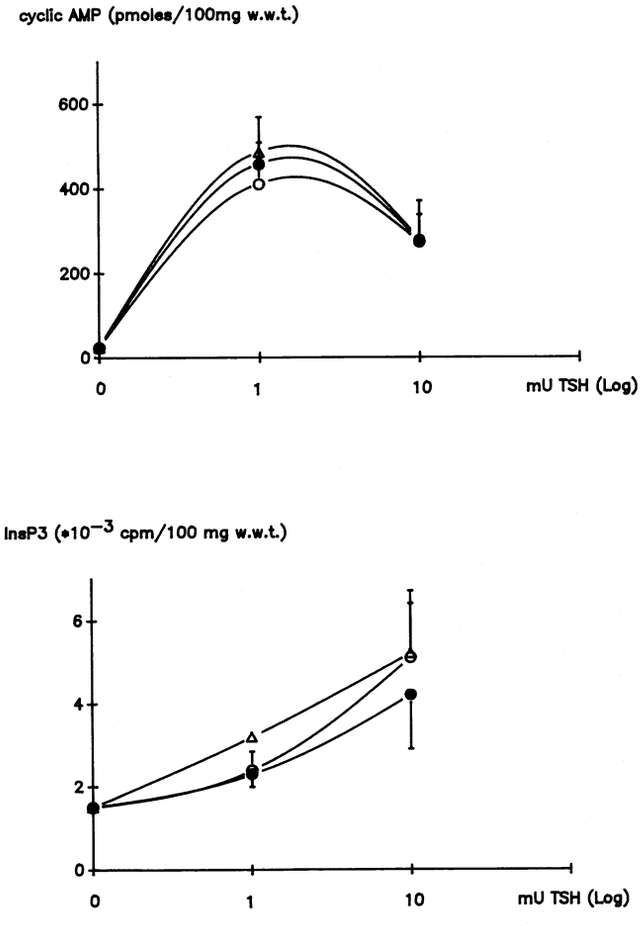

Figure 4. Cyclic AMP and $InsP_3$ concentration-response curves to thytropar (— o —), bovine purified TSH (— ● —) and human purified TSH (— △ —). w.w.t., wet weight tissue. Results are expressed as means±SD of duplicates for cyclic AMP and ±SEM of triplicates for $InsP_3$ determinations.

Effects of Graves' immunoglobulins without TSAb activities on $InsP_3$ generation. None of the 6 ASP lacking TSAb activities as defined in Methods was able to increase $InsP_3$ accumulation in the slices (data not shown).

Discussion

As TSH activates both the cyclic AMP and the phosphatidylinositol-Ca^{2+} cascades in human thyrocytes (3), we addressed the question as to whether TSAb, the antithyrotropin receptor antibodies responsible for Graves' disease, also activate these two pathways. As expected, the thyroid slices system is less sensitive to TSH and TSAb than that using thawed cells in hypotonic medium: some sera elicited a cyclic AMP accumulation in the latter but not in the former system. However, except for a few sera (experiments 14, 17, 18, 19), a good parallelism was achieved between cyclic AMP responses to TSAb in both systems (thawed cells and slices). Thus, the more physiological system, human thyroid slices incubated in vitro can be considered as a good model to investigate TSAb activities as they are usually detected and defined using the more sensitive Rapoport bioassay. In contrast to the FRTL5 cell line and dog thyrocytes in primary culture, in the case of human thyrocytes, it has been demonstrated that TSH activates the PtdIns-Ca^{2+} cascade in the same range of concentrations eliciting other physiological effects (3). These facts raise the possibility of a role for the PtdIns-Ca^{2+} cascade in TSAb action. IgG which bind to thyroid membranes are heterogenous and may contain activities which stimulate or inhibit adenylate cyclase (22, 23). Thus, depending on the titer and/or affinity of these two activities, the response in the TSAb assays could clearly vary with the relative concentrations of the IgG concerned (such variations have been demonstrated by Zakarija and McKenzie [22]). Therefore, we considered it important to assay TSAb samples at different IgG concentrations since, at a single concentration, some may appear negative while positive at a higher or lower concentration. None of the concentrations of the various TSAb tested were able to elicit phospholipase C activation as measured by $InsP_3$ generation. This lack of TSAb effect is not due to a blocking effect of the sera, as the TSH action on the $InsP_3$ generation itself was not inhibited in the presence of these IgG.

These results show that at serum TSAb concentrations usually measured in vivo, no activation of the PtdIns-IP_3 pathway is observed. Preliminary data obtained by B. Corvilain and E. Laurent show that H_2O_2 generation and protein iodination in human thyroid mainly depend on the activation of the PtdIns-$InsP_3$-Ca^{2+} cascade and are not stimulated by agents that increase cyclic AMP (24). Our findings concerning the failure of TSAb to induce any H_2O_2 generation are thus not surprising. This suggests that activation of adenylate cyclase by TSAb also fully accounts for their effects on human thyroid cells. Such a conclusion fits in well with the fact that activation of the cyclic AMP cascade stimulates dog and human thyrocyte secretion, proliferation, and differentiation expression (8, 9) (as observed in Graves' disease), while activation of other cascades, the phorbol ester-protein kinase C pathways, or the growth factor protein-tyrosine kinase pathway, leads to proliferation and loss of differentiated expression and function (9, 25, 26).

As previously reported (27), the effect of TSH on human thyrocytes cyclic AMP accumulation is often biphasic, with a

clear decrease of hormone action at high concentrations. This effect is not due to the presence of contaminants in the bovine TSH preparation used, since it was also observed when using purified bovine TSH (40 IU/mg) or purified human TSH (11 IU/mg). Such a biphasic effect was never observed with TSAb. These facts could explain that at high TSAb concentrations, cyclic AMP accumulations were sometimes higher than the maximal TSH effects (experiments 5 and 8 from Fig. 2). This second discrepancy further distinguishes TSAb and TSH actions. The decreased effect of TSH on cyclic AMP production was generally observed for hormone concentrations eliciting the $InsP_3$ generation effect. It is therefore possible that the stimulation by TSH at a high concentration of the phosphatidyl inositol cascade itself could inhibit cyclic AMP generation. Such an effect could result from direct activation of the inhibitory GTP binding protein N_i (as for norepinephrine α_1 effects), from the inhibition of the activating GTP binding protein N_s by a protein kinase C induced phosphorylation or from Ca^{2+}-calmodulin activation of a cAMP phosphodiesterase.

The existence of IgG stimulating thyroid growth independently of cyclic AMP in the serum of some hyperthyroid or even euthyroid goitrous patients has been suggested (10, 11, 28). However, these results were sometimes based on inadequate methodologies (29, 30) and the experiments were never obtained with human thyroid cells or tissue. It has since been shown that cyclic AMP is the mediator of TSH mitogenic action in the FRTL5 cell line (31) and besides, that the growth promoting action of Graves' sera parallel their action on cyclic AMP accumulation in these cells (14).

In conclusion, our data demonstrate that at concentrations similar to in vivo conditions, Graves' IgG are unable to activate the PtdIns-Ca^{2+} pathway in normal human thyroid tissue. Thus, TSAb do not share all the metabolic actions of TSH on normal human thyroid tissue incubated in vitro. If there were two distinct TSH receptors, one controlling phospholipase C and the other adenylate cyclase activation (such as is the case for many other hormones and neurotransmitters [32–36]), TSAb would only activate the receptor coupled to adenylate cyclase. If there is only one TSH receptor controlling two independent pathways (such as is the case for some muscarinic receptors [37]), TSAb action on this receptor would be incomplete. With the recent cloning of a human TSH receptor the tools now exist to distinguish between these hypotheses (38, 39).

Acknowledgments

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