Heterogeneous signal pathways through TSH receptors in porcine thyroid cells following stimulation with Graves’ immunoglobulin G

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Abstract
Objective: We compared different signal transduction pathways through thyroid stimulating hormone receptor (TSH-R) in porcine thyroid cells (PTC) following stimulation with thyroid stimulating hormone (TSH) and 11 thyroid stimulating immunoglobulin samples (TSI) obtained from patients with Graves’ disease.

Design: Following stimulation with TSI, the level of inositol trisphosphate (IP₃) and [Ca²⁺]ᵢ, as well as the membrane bound protein kinase C (PKC) activity and the intensity of the arachidonic acid (AA) cascade, were determined in PTC.

Results: Seven out of eleven TSI samples activated PTC through IP₃ generation, elevated [Ca²⁺]ᵢ from the intracellular pools, exhibited verapamil-insensitive membrane-bound PKC activation, and enhanced release of [¹⁴C]AA derivates (however, one of the samples was also able to take up Ca²⁺ from the extracellular space). Four out of eleven TSI samples did not activate the phospholipase C (PLC) system in which case the Ca²⁺ signal occurred only in the presence of extracellular Ca²⁺, the membrane bound PKC activation was verapamil sensitive, and in two of these four TSI samples, the AA release was extremely high.

Conclusions: The simultaneous examination of the majority of the known signal pathways using TSI samples showed that TSI samples from different patients activate thyroid cells through different pathways. Their effects differ from that of TSH and, to a certain extent, from each other. The results give a certain new insight into the intracellular mechanisms exerted by TSI.

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Introduction
Recent data from the literature indicate that both thyroid-stimulating hormone (TSH) and thyroid stimulating immunoglobulin (TSI) can activate not only adenyl cyclase (AC), but also phospholipase C (PLC) after binding to thyroid stimulating hormone receptor (TSH-R) (1–4). Based on the experiments of Ginsberg et al. (5), both AC and PLC activation results in activation of phospholipase D which is involved in the stimulation of thyroid cells. It has also been described that TSI alters the [Ca²⁺]ᵢ, the activation and translocation of protein kinase C (PKC), and the activity of phospholipase A₂ (PLA₂) (6, 7). Some authors did not find any increase in cAMP and inositol trisphosphate (IP₃) in human thyroid slices after stimulation with TSI – in contrast to the effect of TSH (8–11).

In the present work we examined simultaneously a wide spectrum of intracellular signalling pathways (measuring IP₃, [Ca²⁺]ᵢ, membrane-bound PKC activity and arachidonic acid (AA) cascade) using cAMP-positive TSI samples obtained from 11 patients with Graves’ disease, and TSH. In blocking experiments, the effect of the Ca²⁺-free medium on the Ca²⁺ signal and the effect of verapamil on membrane-bound PKC activity was measured. The aim of our work was to compare the signalling of TSH and TSI and at the same time to make a comparison between the individual TSI samples.

Subjects and methods

Patients
Eleven female hyperthyroid patients were involved. They were recently diagnosed, untreated, and had a mean age of 28.2 (22–36) years. The hyperthyroidism was proved by measurement of free thyroxine, free triiodothyronine and TSH (sensitive TSH). Anti microsomal antibody, anti thyroglobin antibody, and TSH binding inhibiting immunoglobulin (TBII) levels were
also checked. All clinical and laboratory data indicated Graves’ hyperthyroidism.

**TSI preparation**

Immunoglobulin G (IgG)-enriched fractions were prepared from sera of 11 patients and from pooled sera of 6 female, sex-matched, healthy control subjects according to the method of Bidey et al. (12). The IgG-enriched fractions were divided into aliquots and stored at −20°C until use. IgG from pooled sera of 6 healthy volunteers served as the control.

**Isolation of porcine thyroid cells**

Porcine thyroid cells (PTC) were prepared according to the method of McGrath et al. (13), modified by us (14), under sterile conditions. Briefly, freshly removed thyroids from a local abattoir were subjected to enzymatic digestion. The digested sample was filtered, centrifuged and the pellet was washed vigorously. PTC were re-suspended in RPMI 1640 medium (Gibco Laboratories, Grand Island, NY, USA) containing 5% fetal calf serum and 10⁶ cells/ml. The suspension was incubated in a CO₂ incubator for 24 h. Before the experiments were performed the viability of the cells was checked and proved to be higher than 80% in each experiment.

**cAMP determination**

The cAMP determination was carried out on samples of 10⁷ cells/ml using a cAMP kit (Amersham, Amersham, Bucks, UK) according to the manufacturer’s description. Values were expressed in pmol cAMP/mg protein. All TSI samples provoked significant cAMP production, data for which are not shown in this work.

**Measurement of IP₃**

IP₃ determination was carried out according to the method of Patthy et al. (15). PTC suspension was pre-incubated with 25 mCi myo-[3H]inositol (Amersham, 3.11 TBq/nmol) for 24 h in the presence of LiCl in a shaker. The cell-bound (incorporated) radioactivity was determined, which was at least 50% of the total applied radioactivity. After stimulation, the IP₃ was isolated by reverse phase ion-pair chromatography (16) using inner standard for IP₃ (Amersham). The amount of generated IP₃ was expressed as the percentage of d.p.m. in PTC before stimulation.

**Measurement of [Ca²⁺]i**

The method of McCormach & Cobbold was applied (17). Briefly, PTC were mixed with Indo 1/AM (Calbiochem, La Jolla, CA, USA) from the 1.0 mol/l stock solution. After a preincubation period in a shaker the cells were re-suspended in 1.0 ml Hanks’ balanced salt solution (HBSS) or in Ca²⁺-free HBSS containing 3 mmol/l EGTA. The verapamil (Sigma) pre-treatment was carried out in HBSS containing 1 µmol/l verapamil for 60 min. The [Ca²⁺]i determinations were carried out in a spectrophotometer at 405 and 485 nm. The [Ca²⁺]i levels were calculated according to the given equation.

**Measurement of membrane-bound PKC activity**

PKC activities in the PTC particulate fraction were quantified as described by Bell et al. (18) and modified by Gopalakrishna et al. (19). After stimulation, the PTC were rapidly centrifuged at 4°C and the pellet was re-suspended in ice-cold HEPES-buffered HBSS containing EDTA, EGTA, phenylmethylfluoride (Sigma Chemical Co., St Louis, MO, USA) and leupeptin (Sigma). Then the cells were disrupted ultrasonically and centrifuged at 100 000 g for 45 min (Beckman L5–65B). The pellets containing particulate, i.e. membrane-bound fractions, were solubilised and centrifuged again. The PKC activity in the final supernatants containing the membrane-bound PKC were determined by measuring the [³²P]ATP (Institute of Radiochemical Research, Budapest)-derived [³²P] incorporation into Histone III-S (Sigma). The reaction was initiated by adding labelled ATP, and terminated with ice-cold trichloro-acet-acid (TCA) and bovine serum albumin as carrier 10 min later. Then the precipitate was filtered and washed in ice-cold TCA. The radioactivity was determined with a liquid scintillation counter. The PKC activity was expressed as incorporated [³²P] (pmol/min/mg protein).

**Release of [¹⁴C]AA metabolites**

AA metabolites were determined according to the method of Boraschi et al. (20) using [¹⁴C]AA from Amersham (58.4 mCi/mmol). PTC suspensions were incubated with 0.2 mCi [¹⁴C]AA for 20 min at 37°C and the cell-bound radioactivity was considered as the total activity. Following a rapid centrifugation at 4°C the cells were stimulated, and after 20 min the radioactivity of the supernatants was measured in a scintillation counter. The released radioactivity was calculated as a proportion of the total cell-bound activity.

**Stimulation and blocking experiments, and significance**

PTC were stimulated with 0.1 mU/ml TSH and 2.5 mg/ml protein of TSI samples and controls. In blocking experiments PTC were pre-treated with 1 µmol/l verapamil for 60 min. All experiments were done in triplicate, and the significance was calculated using the Student’s t-test. Significance was set at P<0.01.

**Results**

The results are shown in Table 1. The controls had no effect on the examined parameters. TSH increased IP₃,
The PKC activation by samples 2, 4, 6 and 10 was in cAMP (data not shown). The increase in the IP3 levels and 11 triggered a significantly lower Ca2⁺ accumulation without any increase of IP3 (6, 21). We measured an increased [Ca2⁺]i exerted by all TSI samples in our experiments. Our data are concordant with the findings of Di Paola et al. (24) who examined the effects of Graves’ TSI and described the pathological role of cyclooxygenisation using FRTL-5 cells. In our experiments all TSI samples induced a membrane-bound PKC activation. However, the role of TSI-induced PKC activation is conflicting in the literature (25, 26). The effect of different isoforms of the enzyme which are involved in the activation and in the translocation of PKC probably depends on the ligand used and is species specific (27, 28). Based on these data, different intracellular signal pathways – diacylglycerol production, Ca2⁺ influx, AA cascade – may be involved in the activation of different PKC isoforms.

We examined simultaneously a wide spectrum of intracellular signalling pathways using 11 samples of Graves’ IgG. The number of tested IgG samples is not enough to draw a correct conclusion regarding the intracellular pathways stimulated by TSI. We call attention to the significant AA stimulating effect of TSI detected in each case but undetectable after TSH stimulation. Based on our recent results we emphasise the differences between the single TSI samples, i.e. some of the samples stimulated signal pathways characterised by Ca2⁺ influx through Ca2⁺ channels, by verapamil-sensitive PKC activation, and by failure of PLC activation, while others exerted a signalling process that was TSH-like except for the AA cascade stimulation.

**Acknowledgements**

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**Table 1** Activation of signal pathways in PTCs induced by TSH and cAMP-positive TSI samples. PTCs were stimulated with 2.5 mg protein/ml control sample, with 0.1 mU/ml TSH and with 2.5 mg protein/ml TSI samples from 11 patients with Graves’ disease. In samples marked with V the cells were preincubated with 1 μmol/l verapamil for 60 min before TSI stimulation and the inhibition of membrane-bound PKC activation was more than 50%. All data represent the mean ± S.D. value for AA release in the 11 TSI samples was 56.3 ± 16.74%.

<table>
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<tr>
<th>Stimulus</th>
<th>IP3 (%)</th>
<th>ΔCa2⁺</th>
<th>ΔCa2⁺ in Ca2⁺-free medium</th>
<th>Membrane-bound PKC</th>
<th>[14C]AA release (%)</th>
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<tbody>
<tr>
<td>Control</td>
<td>105 ± 18</td>
<td>9.6 ± 1.13</td>
<td>11.0 ± 3.5</td>
<td>342 ± 92</td>
<td>8.7 ± 2.8</td>
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<td>179 ± 34*</td>
<td>101 ± 38.9*</td>
<td>121 ± 23.5*</td>
<td>1845 ± 156*</td>
<td>11.2 ± 3.9</td>
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<td>TSI samples</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>213 ± 37*</td>
<td>118 ± 54.8*</td>
<td>135 ± 14.4*</td>
<td>2360 ± 340*</td>
<td>49.6 ± 14.3*</td>
</tr>
<tr>
<td>2</td>
<td>114 ± 12</td>
<td>97 ± 33.4*</td>
<td>8 ± 2.1§</td>
<td>1115 ± 347* V</td>
<td>51.8 ± 11.7*</td>
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<tr>
<td>3</td>
<td>165 ± 21*</td>
<td>131 ± 22.1*</td>
<td>127 ± 21.9*</td>
<td>1900 ± 212*</td>
<td>36.2 ± 8.2*</td>
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<tr>
<td>4</td>
<td>102 ± 41</td>
<td>115 ± 38.5*</td>
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<td>1250 ± 296* V</td>
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<td>122 ± 24.6*</td>
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<td>6</td>
<td>108 ± 26</td>
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<td>1385 ± 282* V</td>
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<td>186 ± 7*</td>
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<td>2095 ± 265*</td>
<td>38.8 ± 7.7*</td>
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Mean ± S.D. value for control and stimulated groups; § P < 0.01 difference between control and stimulated groups; † P < 0.01 difference in Ca²⁺ signals between Ca²⁺-containing and Ca²⁺-free medium.
References

2. Deery WJ & Rani S. Protein kinase C activation mimics but does not mediate thyrotropin induced desensitization of adenyl cyclase in cultured dog thyroid cells. Endocrinology 1991 128 2967–2975.

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