

The Role of Protein Kinase C and Its Effect on GHRH in the Regulation of Hormone Secretion by Somatotrophinomas*

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Summary: Phorbol ester-induced release of growth hormone (GH) and prolactin (PRL) from human somatotrophic tumors was examined in vitro. 12-O-tetradecanoyl-phorbol-13-acetate (TPA) strongly stimulated GH and PRL secretion and showed an additive effect on GH secretion if used in combination with GH releasing hormone (GHRH). In contrast, staurosporine exerted a variable inhibitory effect on GH release. There was no correlation between such effects and *gsp* mutations. The findings suggested that TPA doesn't act directly through cAMP signal transduction system.

Key words: somatotrophinoma; phorbol ester; growth hormone releasing hormone; signal transduction

In recent years, the most exciting advance in the research of human pituitary tumors is the discovery that 30%—40% of human pituitary somatotrophinomas carry somatic missense single-base mutations within codons 201 and 227 of the gene for the α -subunit of the stimulatory GTP-binding protein, G_s ($G_s\alpha$). These mutations, termed *gsp* oncogenes, result in constitutive activity of adenylyl cyclase and the subsequent elevations in intracellular cAMP levels are thought to be responsible for tumor growth and excessive growth hormone (GH) secretion^[1,2]. Recent biological and immunohistological studies have also shown that there was an increase in PKC activity and protein expression in human hormone secreting and non-secreting pituitary tumors compared to normal rat or human pituitaries^[3]. Additionally, our recent works have shown that not all human somatotrophinoma cells are responsible, in vitro, to GHRH and growth hormone releasing peptide, GHRP-6, a synthetic hexapeptide, which exerts its effects at least partially via PKC and activation of the phosphatidylinositol (PI) second messenger system^[4], and completely independent of the cAMP system. In view of the above mentioned potential role of PKC in the regulation of hormone secretion, the present study was conducted to use in vitro cell culture to investigate the biochemical characteristics of PKC by human pituitary somatotrophinomas, and the effect of PKC-modulators of hypothalamic peptide GHRH on the regulation of GH and prolactin (PRL) secretion. Moreover, the secretory characteristics of adenomas with the *gsp* mutations were also discussed.

1 MATERIALS AND METHODS

1.1 Culture of Pituitary Cells

The clinical presentation of the 18 patients was evaluated preoperatively by history taking,

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* This project was supported by the National Natural Science Foundation of China (No. 39670736) and Germany GTZ Foundation (PN93.2254-02.100/9517).

physical examination, and determination of serum GH levels before and after OGTT and of GH and PRL levels by endocrine stimulation tests. Freshly resected human somatotrophic pituitary tumors, surgically removed from the patients with acromegaly, were immediately washed in phosphate buffered saline (Oxoid, Basingstocke, UK) containing 100 g/L streptomycin, 200 000 UL penicillin and 2.5 g/L Fungizone (Gibco-BRL, Freiburg, Germany). They were cut into small pieces with scalpels. The tissue fragments were incubated at 37 °C with collagenase (1 g/L, Boehringer, Mannheim, Germany) in an orbital incubator shaker for 30 min to 2 h, depending on the nature of tumors. The dispersed cells were washed and re-suspended in minimum essential medium (MEM) containing 10% (v/v) fetal calf serum (FCS), non-essential amino acids, 20 mmol/L HEPES, 0.75% (w/v) Na_2HCO_3 , 100 g/L streptomycin and 100 000 U/L penicillin (all ingredients from Gibco-BRL) hereafter referred to as culture medium (CM). For each tumor studied, equal aliquots of pituitary cells were distributed into at least 12 glass culture tubes (Labco, Marlow, Buckinghamshire, UK) and allowed to attach and equilibrate during the next 18—24 h. Following this period the cells were washed with CM (2 ml). Experiments were then commenced on the cell cultures.

1.2 Effect of PKC-modulators on GH and PRL Secretion

Equilibrated pituitary cell cultures were washed with modified CM (CM-SFCS), in which the 10% (v/v) FCS was replaced with 5% (v/v) charcoal stripped FCS, prepared as previously described^[5]. Cells were then incubated in either fresh basal CM-SFCS without additives (controls), CM-SFCS containing 0—100 nmol/L 12-O-tetradecanoyl-phorbol-13-acetate (TPA, PKC activator) (Sigma) or 0—100 nmol/L staurosporine (PKC inhibitor) (Sigma). At least 3 cultures were used for each variable. After 4 h or 24 h incubation at 37 °C, media were removed and stored at -20 °C until assayed for GH and PRL content by RIA tech-

nique.

1.3 Influence of PKC-modulators of GHRH on the Regulation of GH Secretion

After equilibration cells were washed with CM-SFCS and then incubated in fresh CM-SFCS (2 ml) containing 2 nmol GHRH (1–44 peptide, Bachem, Heidelberg, Germany) with 100 nmol/L TPA or 100 nmol/L staurosporine. After 4 h (GHRH group) incubation at 37 °C, media were removed and stored at –20 °C until assayed for GH content.

1.4 Identification of gsp Mutations

At operation, a portion of each somatotrophic pituitary tumor was frozen in liquid nitrogen and stored at –80 °C until extracted for DNA, as described previously^[1,2]. A double strand DNA fragment of approximately 400 base-pairs and encompassing codons 201 and 227 of the Gs α gene was generated from each of the DNA preparations by polymerase chain reaction (PCR). The conditions were as follows: somatotrophic tumor-derived DNA (1–3 μ g) was mixed with 5' and 3' amplimers (1 μ mol/L each; 5'-amplimer sequence, 5'-CCACCAGAGGACTCTGAGCCCTCTT-3'; 3'-amplimer sequence, 5'-AGCGTGACCAGC-GACCCTGATCCCT-3'), deoxynucleotide triphosphates (dGTP, dCTP, dATP, dTTP, 200 μ mol/L each), Tris (10 mmol/L), KCl (50 mmol/L), MgCl₂ (1.5 mmol/L), and 2.5 U Taq DNA polymerase (Perkin-Elmer, Uberlingen, Germany) in a total volume of 100 μ l. The reaction was carried through 35 cycles of 96 °C (1 min), 65 °C (2 min), 72 °C (3 min). The PCR products were electrophoresed through a 1% agarose gel (6 cm \times 6 cm) from which the Gs α DNA bands were excised and purified with Qiaex (Diagen, Duusseldorf, Germany). The PCR DNAs were directly sequenced by the di-deoxy method and by using primer annealing reactions, gel electrophoresis and autoradiography. Identification of gsp mutations was made by observing "double-bands" on the sequencing gels^[1–3].

1.5 Statistics

Statistical significance was determined by Student's *t*-tests.

2 RESULTS

2.1 Gsp Oncogenes

Of the 18 somatotrophinomas examined 6 (33%) harbored gsp oncogenes. The mutations were present in codon 201 in 5 cases and in codon 227 in 1 cases. These mutations were previously described and both resulted in constitutive adenylyl cyclase activity^[6].

2.2 In Vitro Effect of TPA and Staurosporine on GH and PRL Secretion

Eighteen human somatotrophic pituitary tumors were examined in vitro by incubation for 4 h. 12 were found to be gsp-negative and 6 gsp-positive.

TPA strongly stimulated GH secretion by all adenomas. Maximal effects were observed with level of 100 nmol/L, which led to 2–30-fold GH secretion. In 4 GH-PRL secreting adenomas the same stimulatory effect of TPA on PRL secretion was observed (fig. 1). But staurosporine of 100 nmol/L (for 4 h) exerted different effects on GH and PRL secretion. In most tumors, significant inhibitory effect of staurosporine on GH and PRL secretion was observed (fig. 2). 17 tumors were treated with TPA and (or) staurosporine incubation for 24 h (11 gsp-negative and 6 gsp-positive tumors). The results were similar to those achieved by the treatment for 4 h. TPA stimulated GH and PRL secretion and staurosporine could also inhibit GH and PRL secretion (fig. 3,4). But the effect of staurosporine was, however, variable, with some tumors exhibiting a maximal effect of two-fold inhibition in GH and PRL secretion, whilst others showing no effect. All above-mentioned effects revealed no correlation between the gsp-negative or -positive groups.

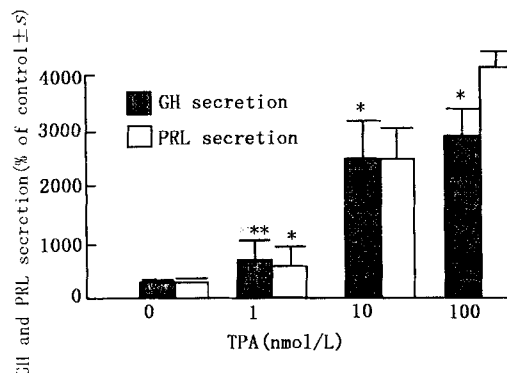


Fig. 1 The effects of TPA on the GH or PRL secretion during 4 h incubation

* $P < 0.05$, ** $P < 0.001$ as compared with control

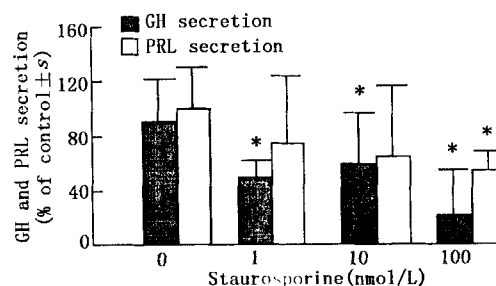


Fig. 2 The effect of Staurosporine on the GH or PRL secretion during 4 h incubation

* $P < 0.05$ as compared with control

2.3 Influence of PKC-modulators on GHRH

In order to determine whether the GHRH induction of GH secretion from pituitary cells is dependent on PKC, we examined the effects of GHRH (incubation for 4 h) alone and in combination with either TPA or staurosporine in some tumors. GHRH alone stimulated GH secretion (1.

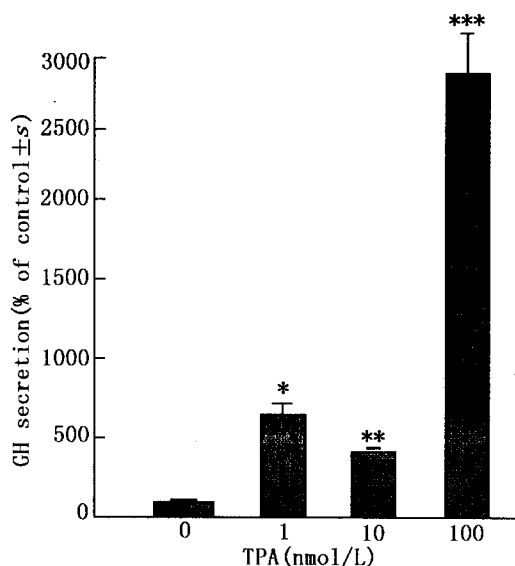


Fig. 3 The effect of TPA on the GH secretion during 24 h incubation
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with control

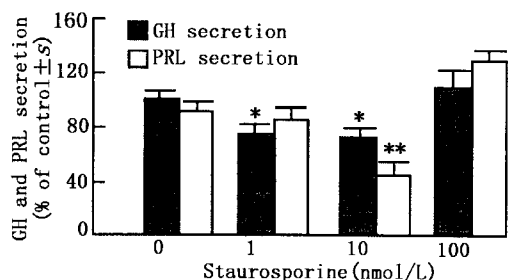


Fig. 4 The doses-dependent effect of Staurosporine on the GH or PRL secretion
* $P < 0.05$, ** $P < 0.001$ as compared with control

4—6.5 fold increase) in 12 out of 18 cases and TPA in all 18 tumors. While combination of GHRH and TPA resulted in additive stimulation of GH. But staurosporine reduced the effect of GHRH substantially (fig. 5). No correlation was exhibited between gsp-negative and gsp-positive tumors.

3 DISCUSSION

Earlier study confirmed the stimulatory effect of TPA on GH but not PRL secretion in cultured rat anterior pituitary cells. But in ovine anterior pituitary cells, TPA, or another PKC-activator, PMA, stimulates both GH and PRL secretion^[7]. Our results showed that TPA stimulated not only GH but also PRL secretion from human somatotrophinomas. While staurosporine by incubation for 4 h showed statistically significant inhibitory effect on GH and PRL secretion only in part of the tumors. GHRH used in combination with TPA exerted an additive effect on GH secretion and the ef-

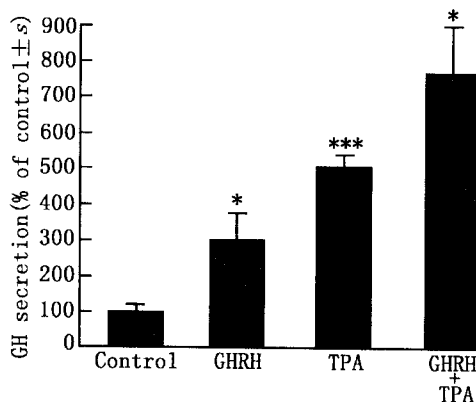


Fig. 5 Influence of PKC-modulator on the effects of GHRH on the GH secretion by somatotrophinomas
* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ as compared with control

fect induced by GHRH could be reduced by staurosporine. It was also demonstrated that TPA stimulated GH and PRL secretion with variable magnitude of the responses from all examined human somatotrophinomas. But GH releasing stimulated by GHRH was observed in only 12 out of 18 adenoma cells and magnitudes of the response was much lower than that of TPA^[8]. In contrast to the effect of TPA, our results with staurosporine revealed a clear difference at different time, doses, and tumors. The cause may be a time-, dose-, and cell-type dependent down-regulation and due to increased rate of degradation^[2].

In order to further elucidate whether PKC-activators involve in regulating GH secretion through cAMP pathway in human pituitary adenomas, we compared the effects of TPA and staurosporine with that of gsp-mutations. The results showed no correlation between gsp-negative or gsp-positive groups. Because in normal human somatotrophs, GHRH stimulates GH secretion through protein kinase A/Gs α /cAMP system and the Gs α -subunit of the heterotrimeric Gs protein is in an inactive GDP-bound state (Gs-GDP). Activation of the bound GDP with GTP results in dissociation of Gs α subunit from the Gs protein complex and activation of adenylyl cyclase. The Gs α -GTP subunit has intrinsic GTPase activity and thus soon returns to the inactive GDP-bound state and a return of adenylyl cyclase activity to normal basal levels ensues. The single-base missense mutations which can occur in codons 201 or 227 of the Gs α gene both result in amino-acid substitutions which abolish the intrinsic GTPase activity of the mature Gs α polypeptide and, consequently, adenylyl cyclase remains permanently active. Under these conditions, GHRH has very little effect on adenylyl cyclase activity^[2, 6] and it is reasonable to expect that GHRH should have reduced or no effect on GH secretion. Despite this, TPA was still able to stimulate GH secretion in the gsp-positive tumors by the same magnitudes in gsp-negative tumors. These observations further demonstrated that TPA acts

not direct via cAMP signal transduction system in human pituitary tumors.

It is well known that, when a ligand binds to its specific receptor on the cell membrane, phospholipase C will be activated, and following hydrolysis of inositol phospholipids (PI), leads to an increase in inositol 1, 4, 5-trisphosphates (IP₃) and diacylglycerol (DAG), both of which are thought to act as "second messengers". IP₃ mobilizes intracellular Ca²⁺, which activates Ca²⁺ and calmodulin dependent PKC (Ca-CaM kinase) and DAG activates PKC. Our previous result showed that TPA not only exerted strong stimulating effect on GH secretion but also inhibitory effect on PI turnover, which is totally different from the mechanism by which GHRP-6 works on GH secretion in somatotrophinoma cells. GHRP-6 stimulates the PI system quickly and in parallel, also a strong GH secretion^[4]. A possible explanation is that GHRP-6 induces PI hydrolysis through its own receptor-mediated pathway while TPA directly activates PKC and then exerts a cellular response. Furthermore, in the presence of high concentration of Ca²⁺, activation of PKC requires less phospholipid degradation, so decreased receptor-induced PI hydrolysis is down-regulated as a result of activation of PKC^[4, 9]. TPA may directly activate PKC and activation of PKC itself may be responsible for the sustained elevation of DAG. The sustained increase of DAG occurs in response to hormone secretion and proliferation. This may explain the short action of GHRP-6 on IP₃ turnover. While TPA directly activates PKC and shows no effect on the early transient PI turnover, but the activated PKC spread its action on the inhibition of PI turnover through down-regulation. A multiple signal transduction system may play an important role in the exact regulation, which awaits further study. In spite of the stimulation of PRL secretion by TPA, it was considered to be an early post-receptor event controlling PRL secretion, without affecting phosphatidyl inositol turnover. The action on PRL secretion seems to be mainly dependent on the redistribution and activation of PKC^[10]. But the endogenous PKC can interfere with the regulation of PRL gene expression induced by both cAMP and Ca²⁺ pathways, two second messengers associated with the action of dopamine in lactotrophic cells, while usually dopamine inhibition of PKC release is mediated by several second messenger pathways, including cAMP, PI, and Ca²⁺.

In summary, by using in vitro cell culture technique, we demonstrated that PKC played an important role in the regulation of GH and PRL se-

cretion in somatotrophinomas. Our results showed that TPA stimulates GH secretion through direct activation of PKC and down-regulation of PI turnover. In contrast, somatotrophinoma cells respond to staurosporine variably, most of them showed no effect on GH inhibition. But the effects of GH stimulation were not correlated with gsp mutations. There was an additive effect if GHRH and TPA were used in combination to stimulate GH release and the effect of GHRH could be reduced by staurosporine. The exact mechanism is unclear.

Acknowledgements

We are grateful to Dr. Eric Adams (University Aston, UK) for his technical support of the DNA sequencing.

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(Received Sept. 3, 1999)