Discontinuous and continuous stimulation of FRTL-5 thyroid cells with bTSH cause different cAMP and nuclear proliferation antigen responses

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Pulsatile TSH secretion has been described in man. We investigated the effect of discontinuous TSH stimulation on FRTL-5 thyroid cells. FRTL-5 monolayers were pulsed with TSH in 4 h incubation periods with alternate 4 h TSH-free intervals, or continuously incubated with TSH. The cAMP production of cells was measured in the supernatant of monolayers. Expression of a nuclear proliferation antigen in FRTL-5 monolayers was determined by a monoclonal antibody (Ki-67) using the alkaline phosphatase-anti-alkaline-phosphatase staining method. The TSH concentration in the stimulation series ranged from 0.01 to 1.0 IU/l medium. Rhythmic cAMP production was observed in both discontinuous and continuous stimulation. With discontinuous stimulation cAMP production peaked after about 24 and 48 h, while in the continuous presence of TSH peaks were observed at 32–40 and 48 h. At all TSH concentrations the effect of discontinuous stimulation was higher than in continuously stimulated cultures. The discontinuous incubation stimulated nuclear proliferation antigen expression of FRTL-5 more intensely and there was a positive correlation with TSH concentration. We conclude that the rhythmic pattern of cAMP production after TSH stimulation is independent of the TSH pulse. The amplitude of stimulation and proliferation of FRTL-5, however, is increased by discontinuous TSH application.

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Highly sensitive immunoradiometric thyrotropin (TSH) assays capable of distinguishing between euthyroid and hyperthyroid TSH levels were used to reveal pulsatile and circadian modulated hormone secretion in healthy men (1). Pulsatile hormone secretion has been recognized for all tropic pituitary hormones both in vivo and in vitro.

The frequency and amplitude of the secretion of hypothalamic releasing hormones probably regulates the release of pituitary hormones. The hypothesis that pulsatile TSH release might be governed by a pulsatile mode of hypothalamic stimulator is supported by the observation that an infusion of nifedipine, which selectively inhibits in vitro the thyroid-releasing hormone (TRH) effect on TSH pulsatile secretion, exerts a comparable effect when it is infused in vivo (2). Moreover, a prolonged exposure to TRH causes homologous pituitary desensitization and decreases TSH secretion (3). Hartnell et al. (4) demonstrated that pulsatile TRH application releases more TSH, triiodothyronine (T3) and thyroxine (T4) than the corresponding amount of TRH administered continuously in man.

In vivo and in vitro gonadotropin-releasing hormone (GnRH) regulates its own receptors, depending on its pulsatile or continuous secretion, which increases or decreases receptor numbers respectively (5). In addition, the investigations of Samuels et al. (6) imply that an underlying unified signal coordinates pulsatile hormone secretion from both gonadotropins and thyrotrophs.

An additional site of investigation of pulsatile regulation is the manner in which pituitary secretion influences peripheral endocrine gland function. Pulsatile compared to continuous stimulation causes higher adenosine 3',5'-cyclic monophosphate (cAMP) and progesterone production in ovarian granulosa cells (7, 8). However, continuous and pulsatile infusion or perfusion of luteinizing hormone (LH) have identical effects on steroidogenic capacity and cAMP output of Leydig cells (9, 10).

The effect on hepatic glucose production of pulsatile glucagon application is superior to the respective continuous infusion even if the hormone saturation is significantly reduced (11, 12).

Scherbaum et al. (13) have shown an autonomous pulsatile T3 and T4 release from isolated porcine thyroid follicles. Hormone output increased in response to TSH stimulation, but release frequency did not change.

The effect of discontinuous versus continuous TSH
stimulation on the thyroid gland or thyroid cells has not thus far been examined. We therefore investigated the influence of these different stimulatory regimes on cAMP accumulation and on the expression of a nuclear proliferation antigen (NPAg) in FRTL-5 (Fischer Rat Thyroid cells in Low serum) cells.

Materials and methods

**cAMP assay**

FRTL-5 cells were routinely cultured in 24 well plates (Nunc) in TSH containing 6H-medium (1U TSH/l) to about 40% confluence. In order to sensitize thyroid cells for the following stimulation, monolayers were deprived of TSH (5H-medium) for 10 days [for procedure in detail see Kohn and Valente (14)].

After stimulation period, supernatant was frozen at -20°C and analyzed afterwards for cAMP using a commercial kit (³H-cAMP, range: 20–320 nmol/l, Amersham). In order to compensate for differences in cell number in each assay well, protein content was measured in the cell debris (Coomassie brilliant blue assay by BioRad).

**NPAg assay**

A nuclear antigen was described in human tumor cells, which is associated with cell proliferation. This antigen is recognized by a monoclonal antibody (Ki-67, Dianova) during the G₁, S, G₂ and M phases of the cell cycle, but is not expressed in Go (15). Recently, we indicated this NPAg in FRTL-5 rat thyroid cells and established a highly sensitive assay (lower threshold: 10 mU bTSH/l) for thyroid stimulating antibody (TSAb) (16). For the stimulation experiments, FRTL-5 cells were plated in cultivation chambers mounted on microscopy slides (LabTek) and then processed as for cAMP assays. NPAg expression was determined by alkaline phosphatase-anti-alkaline phosphatase (APAAP) staining using monoclonal antibody Ki-67. The results are expressed as a percentage of cells NPAg positive, which were quantified by cell counting by light microscopy.

**Treatment protocol**

Discontinuous stimulation was carried out by alternatively stimulating and depriving the monolayers with TSH in 4 h incubation periods. Therefore, TSH was added to the monolayers for 4 h, after which the media were removed and frozen for cAMP assay, the cells were washed twice in PBS and then switched to the TSH-free medium (5H-medium) for 4 h. The cycle was then repeated for a total duration of 52 h. Continuously stimulated cultures also underwent supernatant changes every 4 h, the fresh media containing TSH each time.

Continuous and discontinuous stimulation were carried out using bovine TSH (Organon, 0.5 IU/mg) in concentrations of 0.01, 0.1 and 1.0 U/l. At each timepoint, chamber slides were also fixed in ethanol/chloroform for subsequent NPAg staining. Each test was performed in duplicate, with an additional control without TSH. Mean values of three experiments performed in parallel are shown in the figures.

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**Fig. 1.** cAMP production in FRTL-5 monolayers discontinuously stimulated with bTSH in 4 h periods. The lower part of the figure represents the administration regime of TSH to the wells. N = 3, mean. The coefficient of variation (cv) between the three experiments was between 2 and 14%.

- ◆ 0.01 U bTSH/l; ■ 0.1 U bTSH/l; × 1.0 U bTSH/l; — without TSH.
Results

For both discontinuous and continuous stimulation rhythmic cAMP production was observed. Discontinuous stimulation caused cAMP output peaks at 12, 24 and 48 h, while the continuous presence of TSH caused peaks between 32 and 40 h and at 48 h (Figs 1 and 2).

At all TSH concentrations the discontinuous stimulation effect was higher than in the continuously stimulated cultures.

Cholera toxin, which stimulates the enzyme adenylate cyclase independent of TSH receptors via G protein, also causes a rhythmic cAMP output with peaks at 24, 32 and 44 h (Fig. 2). In non-stimulated monolayers cAMP was only measurable at 24 h (Fig. 1).

In order to evaluate cAMP regulation and indirectly TSH receptor function, we repeated experiments in the presence of isobuthylmethylxanthine (IBMX), which is a highly effective phosphodiesterase inhibitor. We found in both regimes a continuous increase of cAMP concen-

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Fig. 2. cAMP production in FRTL-5 monolayers continuously stimulated with bTSH. The lower part of the figure represents the administration regime of TSH to the wells. N = 3, mean, cv was between 0.2 and 22%. ◆ 0.01 U bTSH/l; ■ 0.1 U bTSH/l; × 1.0 U bTSH/l; — cholera toxin.

Fig. 3. cAMP accumulation in FRTL-5 monolayers discontinuously stimulated with bTSH in the presence of the phosphodiesterase inhibitor IBMX in 4 h intervals. Periodic decline of cAMP amount is caused by TSH deprived intervals. The lower part of the figure represents the administration regime of TSH to the wells. N = 3, mean, cv was between 1 and 20%. ◆ 0.01 U bTSH/l; ■ 0.1 U bTSH/l; × 1.0 U bTSH/l; — without TSH.
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pmol cAMP/mg protein

Fig. 4. cAMP accumulation in FRTL-5 monolayers continuously stimulated with bTSH in the presence of the phosphodiesterase inhibitor IBMX. The lower part of the figure represents the administration regime of TSH to the wells. N = 3, mean, cv was between 0.6 and 22%. • 0.01 U bTSH/l; ■ 0.1 U bTSH/l; × 1.0 U bTSH/l; •••• Forskolin.

tration without pulses. TSH-free periods in the experimental protocol suggest a pulsatile character of cAMP concentration with discontinuous stimulation. But the regression of peaks shows a linear accumulation of cAMP in these cultures (Figs 3 and 4).

A steadily elevated cAMP release with a discreet peak at 24 h was observed in the presence of forskolin, which, like cholera toxin, stimulates the catalytic unit of the adenylate cyclase (Fig. 4). Discontinuous incubation stimulated NPAg expression more intensively (Fig. 5) and a positive correlation between TSH concentration and the amount of NPAg-positive FRTL-5 cells was found (similar data at 0.1 and 1.0 U/l bTSH not shown). Expression and increase of NPAg in non-stimulated monolayers appear to be mediated by additional growth factors, especially insulin and insulin-like growth factor 1 (IGF-1), which are part of the hormone cocktail (5H) or of the applied fetal calf serum respectively (17).

Discussion

We were able to show periodic cAMP production by

Fig. 5. Expression of the nuclear proliferation antigen (NPAg) in FRTL-5 monolayers with discontinuous and continuous stimulation with 0.01 mlU bTSH/ml medium. N = 3, mean. % of cells NPAg positive. □ discontinuous; ● continuous; ■ without TSH.

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stimulating FRTL-5 thyroid cells with bTSH in a discontinuous as well as continuous manner. Stimulation procedures showed different quantity, time interval and amplitude of cAMP pulses. Above all, the amplitude of stimulation and of cell proliferation was increased by discontinuous TSH application, when compared to continuous stimulation.

cAMP release from FRTL-5 monolayers stimulated with cholera toxine also show an episodic pattern. In contrast, inhibition of phosphodiesterase by IBMX, however, largely suppressed cAMP pulsation. The attenuation of responsiveness after continuous stimulation with TSH until 48 h does not appear to be due to desensitization of the adenylate cyclase or down regulation of TSH receptors, since the amount of cAMP in cultures with the phosphodiesterase inhibitor IBMX steadily increased. Indeed, these findings are in contrast to most reports (18–22), but experimental conditions differ largely. The TSH-desensitization and receptor down regulation studies were carried out by the indicated preincubation of thyroid cells with TSH. After a subsequent re-exposure to the hormone cAMP response and [125I]TSH binding were measured. Our data show that cAMP release from FRTL-5 monolayers, measured in 4 h periods, steadily increased until 48 h, when these cultures were stimulated continuously with TSH. Moreover, with continuous stimulation cultures also underwent supernatant changes every 4 h. Figs 3 and 4 possibly indicate a plateau of the cAMP accumulation in the monolayers after 48 h of continuous or discontinuous TSH stimulation. Continuous exposure of FRTL-5 cells to TSH, which induces refractoriness of the cAMP system to subsequent hormone stimulation, does not decrease cAMP accumulation for a prolonged period of 4 h. Of course, our experiment and the above-mentioned experiments were carried out in non-physiological conditions, particularly inhibitors of the phosphodiesterase system were employed.

The pulsatile nature of TSH stimulated cAMP release in FRTL-5 cells is probably conditioned by counter-regulation of the phosphodiesterase. The lower effect of continuous TSH stimulation appears to be due to a process that alters the interaction between the TSH receptor and the guanine nucleotide regulatory component of cyclase (7).

TSH proved to increase DNA synthesis and cell growth in FRTL-5 cells. These processes are clearly cAMP mediated (23, 24). The effect of TSH on cell proliferation in human thyrocytes without the addition of serum has been reported controversially (25, 26). Moreover, these studies are of limited significance because of fibroblast contamination of the primary thyroid cell cultures. Studies with the permanent thyroid cell line FTC-133 from a human follicular thyroid cancer, which is free of fibroblast contamination, showed increased 3H-thymidine incorporation and cell growth at physiological TSH concentrations (27). But these effects in the FTC-133 cells were not mediated by cAMP. In contrast, Roger et al. (28) demonstrated a growth stimulatory effect of cAMP and agonistic substances in primary human thyrocyte cultures. We are reluctant to speculate about the significance in vivo because of these species-specific differences in the growth regulation of thyrocytes and our in vitro experimental design. In conclusion, discontinuous TSH stimulation appears to economize signal transmission for second messenger generation and proliferation in FRTL-5 rat thyroid cells.

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