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TSH Control of PKA Catalytic Subunit Activity in Thyroid Cell Cultures

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The protein expression and the enzyme activity of the catalytic subunit (C) of the cAMP-dependent protein kinases were studied in porcine thyroid cell primary cultures stimulated with two doses of TSH (0.1 mU/ml and 1 mU/ml) for 1 to 3 days. In TSH-stimulated cells the desensitization of the catalytic subunit activity was accompanied by a simultaneous and parallel decrease of its immunoreactivity. The loss of catalytic subunit was rapid and reached its maximum after 1 day of culture. It is similar in the two subcellular compartments: cytosol and particulate extracts. Contrary to the observed loss of the C subunit protein molecules in TSH-stimulated cells, the expression of the Cβ subunit mRNA in these cells was increased fivefold compared to controls, while no significant change was observed on the Cα subunit mRNA. These results suggest that TSH controls the Cβ subunits of PKA at two levels: at the transcriptional level it increases Cβ mRNA expression, and at the translational or posttranslational level TSH decreases the amount and the activity of the Cβ protein molecules.

Key Words: **Cβ subunit; cAMP-dependent protein kinase; mRNA; thyroid cell cultures (porcine); TSH, thyroid stimulating hormone; TSH control of thyroid (cell) gene expression.**

In thyroid cell cultures, TSH controls both the cAMP-dependent (PKA) and the Ca^{2+} PL-dependent (PKC) protein kinases that are mediators of the two principal regulatory cascades involved in the TSH regulation of multiple thyroid functions (1–4). The catalytic activity of cAMP-dependent protein kinases is desensitized in both porcine (1) and dog (2) cultures after exposure of cells to different doses of TSH. Among the two isoforms of cAMP-dependent protein kinases, the PKA I was desensitized preferentially in both porcine and dog cultures. The regulatory subunits (RI and RII) of PKA are also under TSH control which regulates rather the cAMP binding to regulatory subunits than their respective expressions. A greater number of cAMP-binding sites were occupied in TSH-treated cells than in controls. Pools of free RI subunits is in excess compared with C subunits (4).

The desensitization of the catalytic activity, observed in stimulated thyroid cell cultures, but also in some other cell systems when stimulated with their specific regulators (5–7), can be due to the inactivation, upon dissociation of the catalytic activity by specific inhibitors and/or proteases (8). It can be accompanied or not by a simultaneous decrease of protein expression of C subunits.

Two main isoforms of PKA (PKA I and PKA II) are known since 1975 (9) but the complexity of PKA regulation was still enhanced by the discovery of multiple isoforms for both the R- and C-subunits. As many as four R-subunit isoforms (RIα, RIβ, RIIα, RIIβ) and three C-subunit isoforms (Cα, Cβ, Cγ) have been identified in mammals as distinct gene products (10–12). The gene regulation of the catalytic subunit isoforms by TSH is not well established.

Here we studied the kinetics of desensitization of the PKA activity in porcine thyroid cell cultures exposed to TSH for three days. The decrease of the enzyme activity was compared and correlated with the immunoreactivity of catalytic subunit protein molecules evaluated by western blots. We also studied the C-subunit isoforms mRNA expression in cell cultures of control and TSH treated cells.

MATERIALS AND METHODS

Cell cultures. Thyroid cells were isolated from porcine glands by discontinuous trypsinization as previously described (1). Freshly isolated cells were suspended at a concentration of 2×10^6 cells/ml in

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Eagle's minimum essential medium (MEM) purchased from Merieux, Lyon, pH 7.4, containing 10% foetal calf serum, 200 U/ml penicillin and 0.05 mg/ml streptomycin sulfate. They were cultured in petri dishes at 37°C under 5% CO2. TSH (0.1 mU/ml or 1.0 mU/ml) was added from the beginning of the culture period. Bovine TSH 30 IU/mg (NIADDK, bTSH II) and ovine TSH 7.5 IU/mg (NIAMDD, oTSH9) were gifts from NIH (Bethesda, MD). Cultured cells were centrifuged at 250 × g for 5 min and the pellet was rinsed 3 times with 20 ml isotonic Earle-Hepes-buffered saline (pH 7.2). Pellets were frozen for 24 h in dry ice before measuring the protein kinase activity.

Preparation of cytosolic and particulate fractions. Pellets corresponding to 1.4×10^8 cells were homogenized in 4 ml of 20 mM Tris/HCl buffer, pH 7.5, containing 10 mM EGTA, 2 mM EDTA, 250 mM sucrose. Protease inhibitors (4 μg/ml leupeptin and 4 μM PMSF) were added to the homogenization buffer. Homogenates were centrifuged for 60 min at 105,000 × g and the supernatants which represented cytosolic fractions were used to measure the PKA activity. The pellets were resuspended in a half volume of homogenization buffer containing 0.2% (w:v) Triton X 100 and kept at 4°C for one hour with intermittent stirring. The suspensions were then diluted with homogenization buffer to adjust the concentration of the detergent to 0.1% and for 1 h at 105,000 × g. The supernatants of this centrifugation were defined as particulate extracts.

Protein kinase determination. The cAMP-dependant protein kinase activity was measured in 40 μl aliquots of cytosol incubated in a final volume of 400 μl of phosphate buffer, pH 7.4, as previously described (13).

The PKA activity was measured in the presence of 5 μM cAMP (Sigma). Basal activity was measured in the absence of cAMP, but in the presence of the PKA specific peptide inhibitor (Sigma). 0.1 mM [γ-^{32}P]ATP (specific activity of 150–200 cpm/pmol) (Amersham, UK) was added to start the reaction. Incubations were carried out at 30°C for 2 min, spotted onto 2.1 cm diameter filter paper discs (Whatman), and immediately dropped into 10% trichloroacetic acid solution containing 0.1 mM ATP as carrier. Filter paper discs were treated as previously described (13) and the ^{32}P incorporated into proteins were determined by liquid scintillation.

Western blot analysis. Aliquots of subcellular extracts were diluted in 2 volumes of sample buffer (50 mM Tris/HCl, pH 8.8, 2.5% SDS, 20% glycerol, 10% 2-mercaptoethanol and 0.01% bromophenol blue), boiled for 5 min at 100°C, and separated by SDS PAGE. Proteins were then transferred to nitrocellulose sheets (14) and incubated with primary antibody, a gift from Pr. G. Schwoch (University of Gottingen), at the indicated dilutions overnight at 4°C, followed by 1 h incubation at room temperature with anti-rabbit IgG antibody conjugated to horseradish peroxidase. The reaction was revealed using the ECL detection kit (Amersham).

RT-PCR. Poly(A)$^+$ mRNA was isolated from thyroid cell cultures and a random primed single strand cDNA was synthesized by reverse transcriptase and amplified as described in Omri *et al.* (15) using oligonucleotide primers from mouse sequence: 5′GAACCCTCCCCCGAGTAATGC3′ and 5′CGGTGACTCGTCATGATGCGGT3′ were used to amplify the catalytic subunit β, and 5′AGACCCCTCTCACGAATACAGC3′ and 5′CTCTCACCCTTGGTGATGCGGAA3′ were used to amplify the catalytic subunit α (T_M = 60°C).

We obtained a PCR product with the expected size of 122 bp: primers were synthesized by GENSET. Amplifications were performed with 30 cycles in a Perkin-Elmer/Cetus DNA thermal cycler using a cycle of denaturation for 1 min at 94°C, annealing for 1 min at 60°C and extension at 72°C for 1 min. PCR products were separated electrophoretically in 1.3% agarose gel and transferred to nylon membrane using the alkaline transfer method (16). [^{32}P]dATP-labeled oligonucleotide used as probes to detect the correct PCR products were 5′TTTGGAAGAGTCATGTTGGTG3′ and 5′TTTGGGCGAGTGATGCTGGTG3′ for the catalytic subunits β and α, respectively. A GAPDH probe was used as control for uniform RNA content.

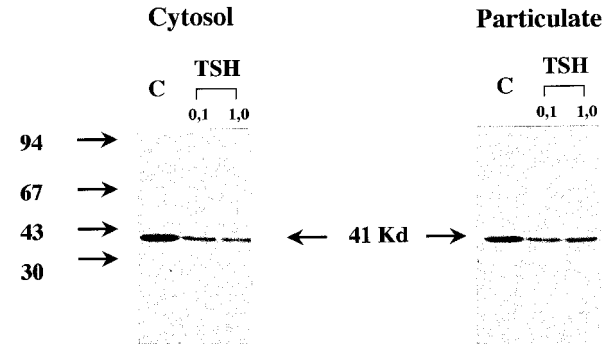

FIG. 1. Immunoblots of PKA catalytic subunit in cytosols and particulate extracts in control (C) and TSH-stimulated cells (TSH, 0.1 and 1 mU/ml) cultured for 2 days. Samples of cytosol (20 μg DNA equivalent) and particulate extracts (40 μg DNA equivalent) were analysed as described under Materials and Methods. Nitrocellulose sheets were incubated with anti-PKA C-subunit antibody used at 1:4000 dilution overnight at 4°C. The second antibody was used at 1:30,000 dilution for 1 h at room temperature.

Expression of results. Protein kinase activities are expressed as pmol of ^{32}P incorporated into histones per equivalent of 100 μg DNA, per min of incubation. DNA content was measured in aliquots of homogenates as described by Groyer and Robel (17). Results are expressed as percent of the total activity relatively to the control cell activity. All results are expressed as mean ± SEM of the indicated number of experiments.

RESULTS

Immunoreactivity of catalytic subunits of PKA. Western blot analysis revealed only one major band of 41 kDa, in both cytosol and particulate extracts, and occasionally traces of one smaller band which was found only in cytosols (Fig. 1). In the two subcellular compartments from TSH treated cells, the immunoreactivity of the catalytic subunit was decreased in similar manner for both TSH doses studied. The faint band found in the cytosol was apparently not modified in stimulated cells (Fig. 1). The loss of C subunit immunoreactivity was quantified by scanning the films and was compared with the loss of the enzyme activity measured in parallel assays. The decrease of C subunit enzyme activity and its immunoreactivity were of the same magnitude although slightly smaller in cells exposed to 0.1 mU/ml TSH than in cells exposed to 1 mU/ml TSH, 40% and 50% respectively. For each dose of TSH, they were identical in the two cellular compartments: cytosol and particulate extract (Fig. 2).

Time course of PKA activity and C subunit protein expression were studied during exposure of cultured cells to TSH for three days (Fig. 2). As in previous experiments, in TSH stimulated cells, the C-subunit loss observed by enzymatic activity and immunoreactivity experiments were simultaneous and parallel in the two subcellular compartments. A maximum is already reached after one day culture and maintained at the same level throughout the culture period.

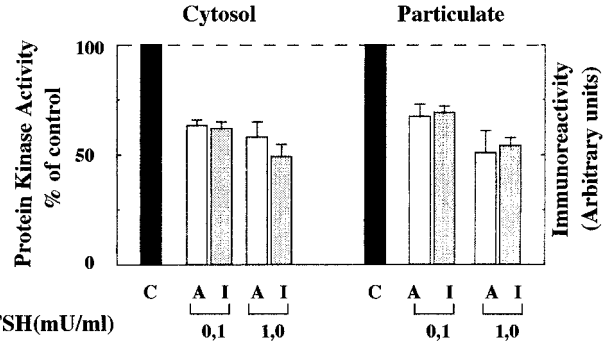

FIG. 2. Graphic presentation of enzymatic activity (open bars, A) and protein amounts (hatched bars, I) of the PKA catalytic subunits in cytosols and particulate extracts from cells stimulated with TSH (0.1 mU/ml and 1.0 U/ml) for 2 days. Enzyme activity was evaluated by direct measurements in aliquots of cytosols (20 μg DNA equivalent) and particulate extracts (40 μg DNA equivalent). Protein amounts were quantified by scanning immunoblots prepared as in Fig. 1. Results are expressed as percentages of controls (black bar). Each point represents the mean ± SEM of 6 independent experiments.

RT-PCR. The amplification of the specific 5′ region of the Cα and Cβ isoforms was realized by reverse transcription-PCR from poly(A)$^+$ mRNA prepared from porcine thyroid cell cultures. In cell cultures, the expected fragment of 122 bp was obtained with PKA C isoform amplification. This 122 bp fragment hybridized with the specific probe described in material and methods (Fig. 3). Experiments were performed, in parallel, with cultured thyroid cells exposed to TSH for three days. Our results show that TSH treatment does not affect the amount of Cα isoform RNA but increases the transcription of the Cβ isoform mRNA by five fold as quantified by scanning (Fig. 3).

DISCUSSION

The complexity of PKA regulation is enhanced by the presence of multiple isoforms for both the R- and C-subunits. As many as four R-subunit isoforms (RIα, RIβ, RIIα and RIIβ) (11) and three C-subunit isoforms (Cα, Cβ, and Cγ) have been identified in mammals as distinct gene products (12). In addition, two putative alternative spliced products of the Cβ gene have been identified (18). Allowing all possible combinations of R- and C- subunits, at least 12 PKA holoenzymes could be formed; some of them have been identified and separated in tissues and transfected mammalian cells (12).

Concerning the catalytic subunits, it is considered in general that Cα-isoforms are constitutively expressed in all tissues while the other two isotypes (Cβ and Cγ) are expressed in a tissue specific manner. The catalytic subunit Cγ is expressed only in testis (12).

The present results and those reported previously (1) clearly show that the global catalytic activity of PKAs is down regulated in parallel in the two subcellular compartments (cytosol and particulate extract), in cells cultured in the presence of TSH. A similar desensitization was also described in several other cell systems. After a brief increase of intracellular cAMP, the desensitization is reversible (5), while after a sustained increase in the cAMP it is irreversible (down-regulated) (6, 7). Results of radioimmunological assays, reported here, clearly indicate that desensitization in our cell system is due to the loss of enzyme molecules as it was described in some other cell systems (6, 8). Since we observed a parallel decrease of the PKA catalytic activity in the both cellular compartments, we can exclude a nuclear translocation as described by Dremier and al in dog thyroid cells (19). On the other hand it is known that with respect to holoenzyme, the free catalytic subunits in stimulated cells are more sensitive to the proteolytic degradation (8) by the specific C-subunit protease (20), which thus accelerates their turnover (21) and that is consistent with the decrease of protein expression of C subunits observed in our results.

Contrary to the decrease of the number of catalytic subunit protein molecules, the parallel study on the catalytic subunit mRNA shows that only the expression of Cβ isoform is enhanced by five fold in TSH-stimulated cells compared to controls. Similar phenomenon was reported in a neoplastic B Cell Line (Reh) in which forskolin or cAMP decreased catalytic activity and increased the Cα mRNA level (22). These authors raised a question whether increased mRNA level may be secondary to the decrease in Cα protein, and suggested an autoregulatory mechanism at the gene level which could serve to maintain the level of Cα within certain limits.

In thyroid glands, TSH exerts similarly a double control of the thyroglobulin expression at both the transcriptional and post-translational level. In TSH stimulated thyroids, the expression of thyroglobulin mRNA is increased (23). At the same time the degradation and the turnover of the neosynthetized thyroglobulin is accelerated, which leads to its almost complete depletion in chronically stimulated glands (24).

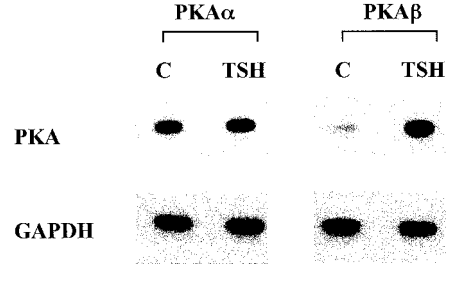

FIG. 3. Southern hybridization of RT-PCR products. The fragment of 122 bp corresponding to Cα (left) or Cβ (right) sequences is detected in control and in TSH-stimulated thyroid cells cultures.

In conclusion, our results reported here suggest that TSH controls the Cβ subunits of PKA at two levels: at the transcriptional level it increases its mRNA expression and/or at the translational or post-translational level TSH decreases the amount and the activity of the Cβ protein molecules.

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