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Thyroid Stimulating Hormone Upregulates Secretion of Cathepsin B from Thyroid Epithelial Cells

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Constant levels of thyroid hormones in the blood are principal requirements for normal vertebrate development. Their release depends on the regulated proteolysis of thyroglobulin which is extracellularly stored in the follicle lumen under resting conditions. Thyroglobulin is proteolytically degraded to a major part in lysosomes, but in part also extracellularly leading to the release of thyroxine. Extracellularly occurring lysosomal enzymes are most probably involved in the proteolytic release of thyroxine. In this study we have analyzed the secretion of cathepsin B by thyroid follicle cells (primary cells as well as FRTL-5 cells) and its regulation by thyroid stimulating hormone, which stimulated the secretory release of the proenzyme as well as of mature cathepsin B. Within one to two hours of stimulation with thyroid stimulating hormone, the cathepsin B activity associated with the plasma membrane increased significantly. This increase correlated closely with the localization of lysosomes in close proximity to the plasma membrane of cultured thyrocytes as well as with the thyroxine liberating activity of thyrocyte secretion media. These observations indicate that thyroid stimulating hormone induces the secretion of cathepsin B, which contributes to the extracellular release of thyroxine by thyrocytes.

Key words: Cathepsin B / Cysteine proteinases / Epithelial cells / Extracellular proteolysis / FRTL-5 Cells / Thyroglobulin.

Introduction

Constant levels of thyroid hormones in the blood are essential for normal development and maintenance of vertebrate organisms (Fujita, 1988). The synthesis of thyroid hormones and their release into the circulation are controlled by thyroid stimulating hormone (TSH; for reviews see Wollman, 1969; Dumont *et al.*, 1992).

TSH is a glycoprotein hormone which is derived from pituitary cells, and which exerts pleiotropic effects on the thyroid gland (Wollman, 1969; Dumont *et al.*, 1992). The primary target cells for TSH are thyroid epithelial cells bearing TSH receptors in their basolateral plasma membranes. TSH stimulates an acute release of thyroid hormones from the thyroid into the blood stream within a few hours (Wollman, 1969; Ekholm, 1990). This short-term effect of TSH is most probably mediated by a rise in intracellular free Ca^{2+}-levels (Chiovato and Pinchera, 1991; Dumont *et al.*, 1992), and leads to the proteolytic degradation of thyroglobulin (Tg; Mercken *et al.*, 1985), the macromolecular precursor of thyroid hormones, for their liberation. Thyroxine (T_4) is biologically inactive, but it is the main hormone released from the thyroid into the circulation (Ekholm, 1990). In contrast, the biologically active thyroid hormone, triiodothyronine (T_3), is predominantly formed through extrathyroidal deiodination of T_4 by target cells such as hepatocytes (Ekholm, 1990; Sharifi and St. Germain, 1992).

TSH binding to its receptors on thyroid epithelial cells also triggers an increase of intracellular cyclic AMP (cAMP) levels (Chabaud *et al.*, 1988; Chambard *et al.*, 1990). The activation of the adenylate cyclase system by TSH results in the upregulation of Tg synthesis on the transcriptional and on the translational level (Dumont *et al.*, 1992). Therefore, TSH promotes the differentiation of thyroid epithelial cells in that it directly affects biosynthesis of the prohormone Tg. In addition, TSH is also believed to stimulate Tg secretion into the extracellular lumen of thyroid follicles (Ekholm, 1990). Thus, the TSH-mediated stimulation of Tg synthesis and of its secretion is responsible for a marked increase of luminal Tg, and is referred to as the so-called long-term TSH-effect. Within the extracellular lumen of thyroid follicles, Tg is stored at high protein concentrations of up to 800 mg/ml (Hayden *et al.*, 1970; Smeds, 1972). Furthermore, extracellularly stored Tg is compacted by covalent cross-linkages, and thereby large aggregates with diameters of 20–120 µm, the so-called Tg globules, are formed (Herzog *et al.*, 1992; Berndorfer *et al.*, 1996; Baudry *et al.*, 1998; Klein *et al.*, 2000; Saber-Lichtenberg *et al.*, 2000).

Recently, we have proposed that cysteine proteinases like the cathepsins B and K are involved in the regulation of the size of the luminal content of thyroid follicles, because both enzymes have the capability to mediate extracellular proteolysis of Tg (Brix *et al.*, 1996, 2001; Tepel *et al.*, 2000). Interestingly, the subcellular localization of cathepsins B and K in thyroid epithelial cells is not restricted to lysosomes. Both cysteine proteinases were also detected

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within the extracellular lumen of thyroid follicles as well as in association with the apical plasma membrane of thyrocytes (Brix *et al.*, 1996; Tepel *et al.*, 2000). Furthermore, cathepsins B and K were shown to liberate T_4 from its precursor Tg in physiologically relevant conditions (Brix *et al.*, 1996; Tepel *et al.*, 2000). Hence, extracellularly occurring lysosomal enzymes are most probably crucial for proper thyroid function in that they mediate the liberation of T_4 by extracellular proteolysis of Tg (Brix *et al.*, 2001).

Here we have analyzed the regulation of lysosomal enzyme secretion from thyroid epithelial cells. As cellular models, primary porcine thyrocytes and a rat thyroid epithelial cell line, *i.e.* FRTL-5 cells (Ambesi-Impiombato *et al.*, 1980), were used. Within short time intervals, TSH upregulated the amounts of extracellularly occurring cathepsin B in both cellular systems. Besides secretion of procathepsin B *via* the constitutive secretory pathway, significant amounts of the mature forms of the protease were released, suggesting that TSH stimulated exocytosis of cathepsin B from lysosomes. It was also found that TSH increased the cathepsin B activity associated with the plasma membranes of thyrocytes within 1–2 h of TSH treatment. A similar time course was observed for TSH-stimulated T_4 liberation by extracellular proteolysis. Our results indicated that TSH upregulates cathepsin B secretion, thereby increasing the potency of thyroid epithelial cells to degrade Tg by extracellular means.

Results

TSH Induces Upregulation of T_4 Liberation by Extracellular Proteolysis of Tg

Primary cultured thyrocytes from porcine origin were incubated for 1–13.5 h with culture medium containing 50 μU/ml TSH. After the indicated time intervals, secretion media and lysosomal fractions were prepared. To test for the potency of secreted or lysosomal cysteine proteinases to liberate T_4 or T_3, secretion media or lysosomal fractions were reactivated with cysteine and incubated with the prohormone Tg for 30 min at 37 °C. The reactions were carried out at pH 7.2 for secretion media and at pH 5.0 for lysosomal fractions in order to mimick the expected pH-conditions of extracellular and intracellular proteolysis, respectively. As determined by radioimmunoassays, the extent of T_3 liberation by extracellular proteolysis of Tg was neglectable (Figure 1A, circles). However, a constant extent of T_3 liberation was observed when lysosomal fractions of TSH stimulated thyrocytes were incubated with Tg (Figure 1B, circles). In contrast, T_4 liberation by secreted cysteine proteinases was detectable, and reached a maximum after 2 h of TSH stimulation (Figure 1A, squares), whereas the potency of lysosomal proteases to liberate T_4 from Tg at acidic pH was not altered by TSH stimulation over the time interval tested (Figure 1B, squares). The results indicate that TSH upregulates the extracellular T_4 liberating activity of thyrocytes within 2 h. Furthermore, the results suggest the secretion of Tg-degrading enzymes as a response to TSH stimulation. Because the thyroid hormone liberation assays were carried out under conditions of reactivated cysteine proteinases, we thought to further study cathepsin B as a major cysteine proteinase expressed in thyrocytes in more detail.

TSH-Induced Redistribution of Cathepsin B-Containing Lysosomes

The rat thyrocyte cell line FRTL-5 possesses TSH receptors at the plasma membrane. FRTL-5 cells were incubated for 4 h without (Figure 2A) or with increasing

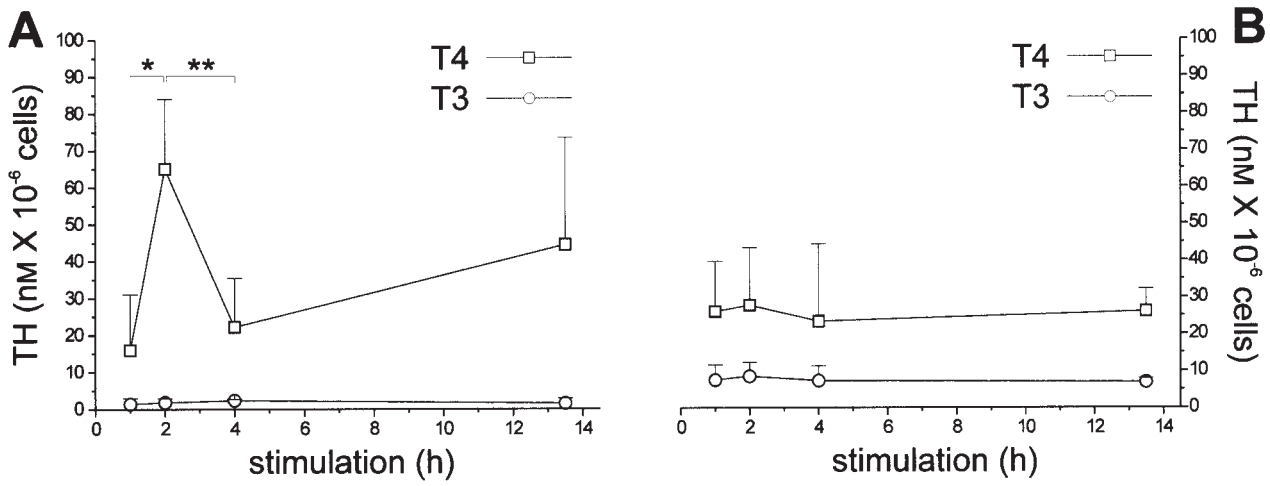

Fig. 1 T_4 Liberation by Extracellular Tg Proteolysis Is Stimulated by TSH.
Culture supernatants (A) and lysosomal extracts (B) of primary cultured porcine thyrocytes were analyzed for the T_3 and T_4 liberating activity after stimulation with 50 μU/ml TSH for the indicated time intervals. Culture media and lysosomal fractions were incubated with exogenous Tg at pH 7.2 and pH 5.0, respectively. The extent of T_3 (circles) and T_4 liberation (squares) from Tg within 30 min was determined by radioimmunoassay. Values for the amounts of thyroid hormones (TH) per 10^6 cells are given as mean + standard deviation. Levels of significance are indicated as * for $p<0.05$ and as ** for $p<0.01$.

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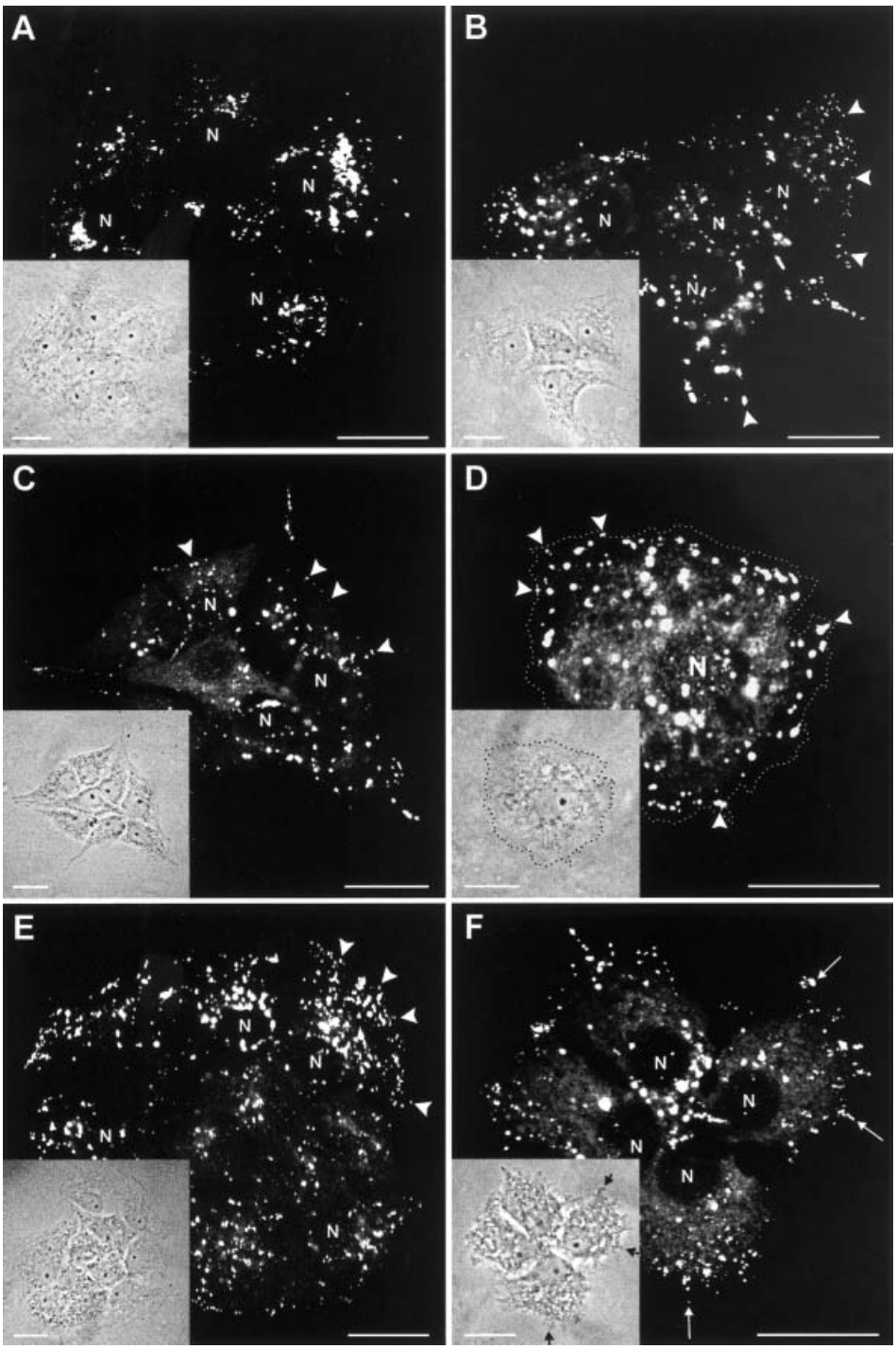

Fig. 2 TSH Affects the Distribution of Cathepsin B Containing Vesicles in FRTL-5 Cells in a Dose-Dependent Fashion.
Confocal fluorescence and corresponding phase contrast micrographs (insets) of FRTL-5 cells after incubation with culture medium without or with TSH for 4 h at 37 °C. After fixation and permeabilization, cells were immunolabeled with anti-rat cathepsin B and Cy3-conjugated secondary antibodies. FRTL-5 cells were incubated with 5H-medium without TSH (A), or supplemented with 3 μU/ml (B), 30 μU/ml (C and D), or 300 μU/ml TSH (E). For chronic TSH stimulation, FRTL-5 cells were grown in 6H-medium containing 100 mU/ml TSH (F). Arrowheads indicate lysosomes in close proximity to the plasma membrane. In chronically TSH stimulated FRTL-5 cells (F), cathepsin B positive lysosomes were often detected within cellular extensions (arrows). Bars, 20 μm; N, nuclei; the dotted lines in (D) indicate the position of the cell surface.

amounts of TSH in the culture medium (Figure 2B-E), and were compared to FRTL-5 cells which were continuously stimulated with TSH by growth in 6H-medium (Figure 2F). Under non-stimulating conditions, FRTL-5 cells exhibited only few cellular extensions (Figure 2A, inset), whereas FRTL-5 cells chronically stimulated with TSH were characterized by numerous cellular extensions (Figure 2F, inset). Immunolabeled cathepsin B was detected within

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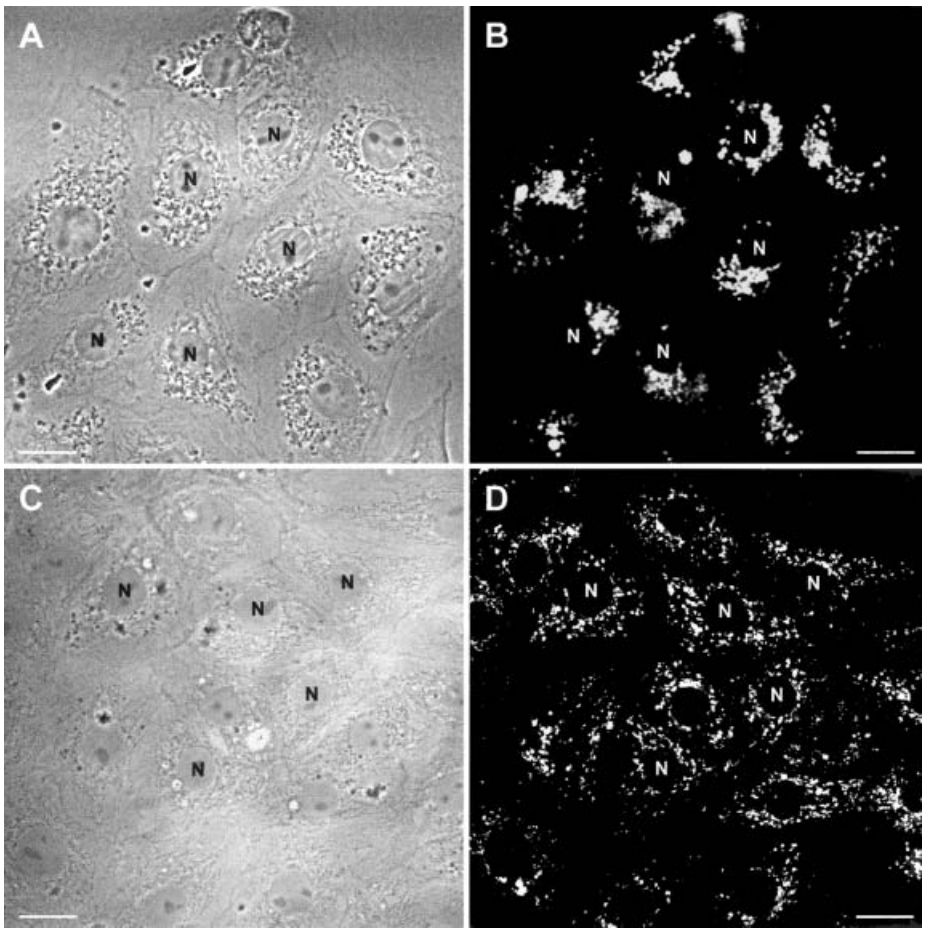

Fig. 3 TSH-Induced Redistribution of Lysosomes in Porcine Thyrocytes.
Confocal fluorescence (B and D) and corresponding phase contrast micrographs (A and C) of primary cultured porcine thyroid epithelial cells after incubation in culture medium without (A and B) or with 50 µU/ml TSH (C and D) for 2 h at 37 °C. Cells were fixed and permeabilized before cathepsin B immunolabeling with anti-human cathepsin B and TRITC-conjugated secondary antibodies. Bars, 20 µm; N, nuclei.

vesicles of crescent-shaped distribution in the perinuclear region of non-stimulated FRTL-5 cells (Figure 2A). Stimulation of the cells with increasing amounts of TSH resulted in a redistribution of cathepsin B containing vesicles from the perinuclear region to the cell periphery (Figure 2 B-E). Such lysosomes of stimulated FRTL-5 cells were often found in close proximity to the plasma membrane (Figure 2 B-E, arrowheads). Under conditions of chronical TSH exposure, FRTL-5 cells acquired cellular extensions bearing numerous cathepsin B containing lysosomes (Figure 2F, arrows). Because the cellular morphology was only slightly altered and because the effect of TSH-induced redistribution of lysosomes was most obvious at a concentration of 30 – 300 µU/ml TSH, this range was chosen for further experiments.

FRTL-5 cells exhibit most, but not all features typical for thyroid epithelial cells. For instance, FRTL-5 cells grow as isolated cells with loose cell-cell contacts and in a less polarized fashion as compared to thyrocytes *in vivo*. Therefore, primary cultured epithelial cells from porcine thyroids were incubated with or without 50 µU/ml TSH before immunolabeling of cathepsin B. Non-stimulated porcine thyrocytes contained numerous lysosomes concentrated within the perinuclear region (Figure 3A and B). After 2 h of TSH stimulation, lysosomes were distributed throughout the cells, which were otherwise without striking morphological alterations (Figure 3C and D).

These results indicate that TSH induces a redistribution of lysosomes from the perinuclear region to the cell periphery of thyroid epithelial cells. This redistribution of lysosomes and, most importantly, their close proximity to the plasma membrane suggest that lysosomes might eventually fuse with the plasma membrane under conditions of acute TSH stimulation, which would result in the release of lysosomal enzymes into the extracellular space.

TSH-Induced Secretion of Mature Cathepsin B

Thyroid epithelial cells of porcine origin were biosynthetically labeled with [^{35}S]-methionine/[^{35}S]-cysteine for 1 h (Figure 4A) or for 2 d (Figure 4B). Cells were washed and

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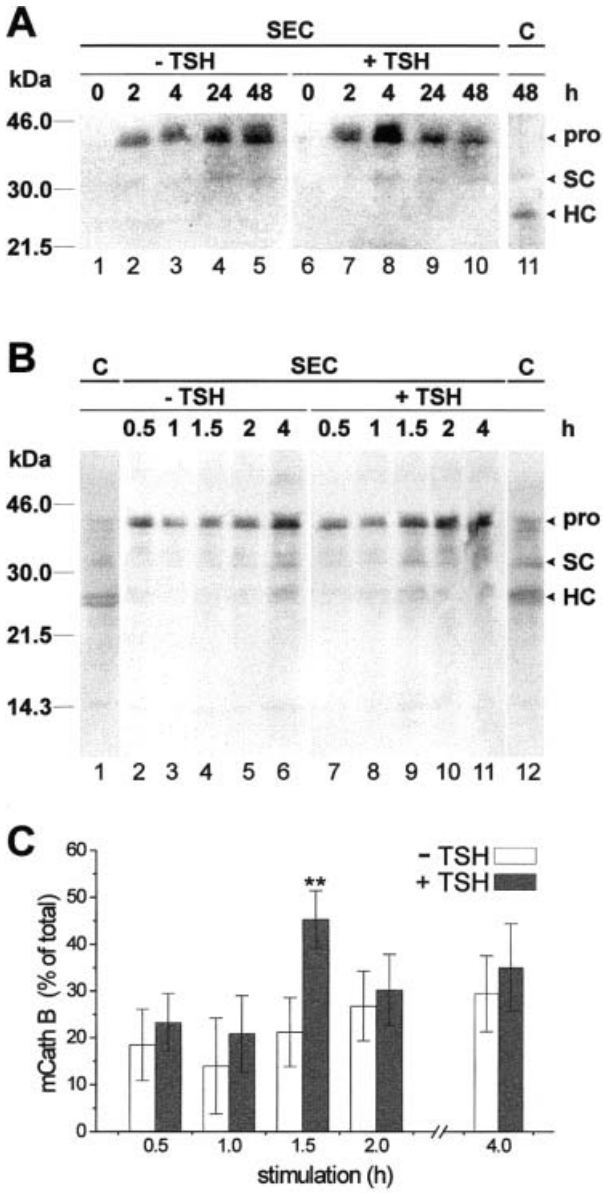

Fig. 4 Secretion of Mature Forms of Cathepsin B from Porcine Thyrocytes Is Upregulated by TSH.

Primary cultured porcine thyrocytes were biosynthetically labeled for 1 h (A) or for 2 d (B) with culture medium containing [^{35}S]-methionine/[^{35}S]-cysteine prior to a chase without (-TSH) or with 50 μU/ml TSH (+TSH) for the indicated time intervals. Immunoprecipitates with anti-human cathepsin B antibodies from culture supernatants (SEC) or cell lysates (C) were separated on 15% SDS-gels. Autoradiography of the gels (A and B) and densitometric evaluation (C) of the bands representing mature forms of secreted cathepsin B are shown. The positions of the proform of cathepsin B (pro) as well as mature cathepsin B, *i.e.* the single-chain form (SC) and the heavy chain (HC) of the two-chain form, and the positions of molecular mass markers are indicated in the margins of (A and B). Densitometric analysis (C) of single-chain plus heavy chain cathepsin B secreted from steady-state labeled thyroid epithelial cells (see B) is expressed as percent of total mature forms of cathepsin B being present in culture supernatants plus cell lysates at the indicated time intervals. Two gels, each, from two independent experiments were analyzed, and values are given as mean ± standard deviation. Levels of significance are indicated as ** for $p<0.01$.

then chased with culture medium supplemented without or with 50 μU/ml TSH. Cathepsin B was immunoprecipitated from culture supernatants and cell lysates with rabbit anti-human cathepsin B antibodies. Immunoprecipitated proteins were separated by SDS-PAGE under reducing conditions followed by autoradiography (Figure 4A and B), and densitometric analysis (Figure 4C).

The main form of cathepsin B immunoprecipitated from the culture supernatants of thyroid epithelial cells after pulse-labeling and under steady-state conditions was the secreted proenzyme (Figure 4A and B, pro). However, increasing amounts of single-chain (SC) cathepsin B were detectable after 1 h pulse-labeling and chasing cells in media without TSH for time intervals of up to 48 h (Fig-

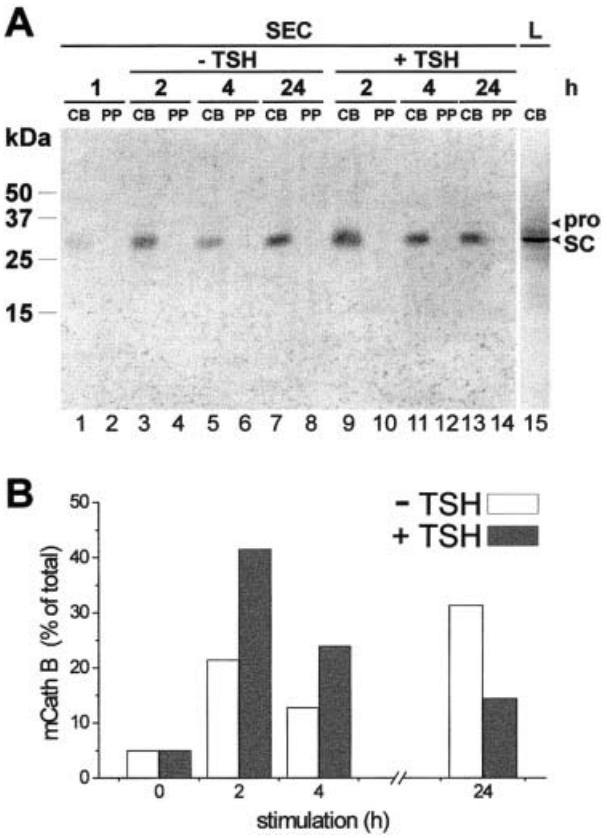

Fig. 5 FRTL-5 Cells Secrete Newly Synthesized Mature Cathepsin B.

FRTL-5 cells were pulse-labeled with [^{35}S]-methionine/[^{35}S]-cysteine in 5H-medium for 1 h at 37 °C. Chase was in 5H-medium without (-TSH) or supplemented with 50 μU/ml TSH (+TSH) for the indicated time intervals. Cathepsin B was immunoprecipitated with anti-rat cathepsin B (CB) or anti-rat cathepsin B propeptide (PP) antibodies from culture supernatants (SEC) or lysates of lysosomes (L). Immunoprecipitates were separated on 12.5% SDS-gels. Autoradiography of the gel (A) and densitometric evaluation (B) of the bands representing secreted single-chain cathepsin B are shown. The positions of procathepsin B (pro), single-chain mature cathepsin B (SC), and of molecular mass markers are indicated in the margin (A). Densitometric analysis (B) of secreted single-chain cathepsin B is expressed as percent of total cathepsin B present in culture supernatants plus lysosomal extracts at the indicated time intervals.

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ure 4A, SC, -TSH). Chasing of pulse-labeled thyroid epithelial cells in media containing TSH resulted in an increase of the amounts of secreted procathepsin B and of the single-chain form within 4 h (Figure 4A, +TSH), suggesting that TSH upregulated the secretion of newly synthesized cathepsin B. Under steady-state conditions, single-chain (SC) and the heavy chain of two-chain mature cathepsin B (HC) were detectable within the culture supernatants during all time intervals analyzed (Figure 4B), suggesting continuous secretion of mature forms of cathepsin B. The amounts of secreted mature forms of cathepsin B showed an almost linear increase in non-stimulated cells (Figure 4C, white bars). Upon TSH stimulation of the cells, the amounts of the secreted mature forms of cathepsin B showed a sharp increase after 1.5 h of stimulation (Figure 4C, grey bars, 1.5 h) reaching a level of 45% of the total mature cathepsin B. These results thus indicate that secretion of mature cathepsin B is a fast process and that TSH significantly stimulates this process by a factor of about 2 (Figure 4C, compare grey bars with white bars at 1.5 h).

To analyze whether FRTL-5 cells were similarly able to secrete cathepsin B, they were pulse-labeled with [^{35}S]-methionine/[^{35}S]-cysteine for 1 h, and then chased in 5H-medium without or with the addition of 50 µU/ml TSH for the indicated time intervals of up to 24 h (Figure 5). Cathepsin B was immunoprecipitated from the culture supernatants of non-stimulated or stimulated FRTL-5 cells, immunoprecipitates were separated by SDS-gel-electrophoresis, and bands representing cathepsin B were visualized by autoradiography. Pulse-labeling for 1 h resulted in the incorporation of radioactive [^{35}S]-methionine/[^{35}S]-cysteine into newly synthesized cathepsin B. In the immunoprecipitates from culture supernatants of non-stimulated or stimulated FRTL-5 cells, only bands were detected which represented the single chain form of mature cathepsin B (Figure 5A, SEC; CB). No bands were detectable when culture supernatants were immunoprecipitated with antibodies against the proform of cathepsin B (Figure 5A, SEC; PP). The absence of secreted procathepsin B is most probably explained by the notion that maturation of cathepsin B appeared to be a very fast process in FRTL-5 cells. Accordingly, only faint amounts of procathepsin B as compared with much higher amounts of single-chain cathepsin B were detectable in immunoprecipitates from lysosomes after 1 h pulse-labeling of FRTL-5 cells (Figure 5A, lane 15). Under non-stimulating conditions, *i.e.* chasing in 5H-medium, sin-

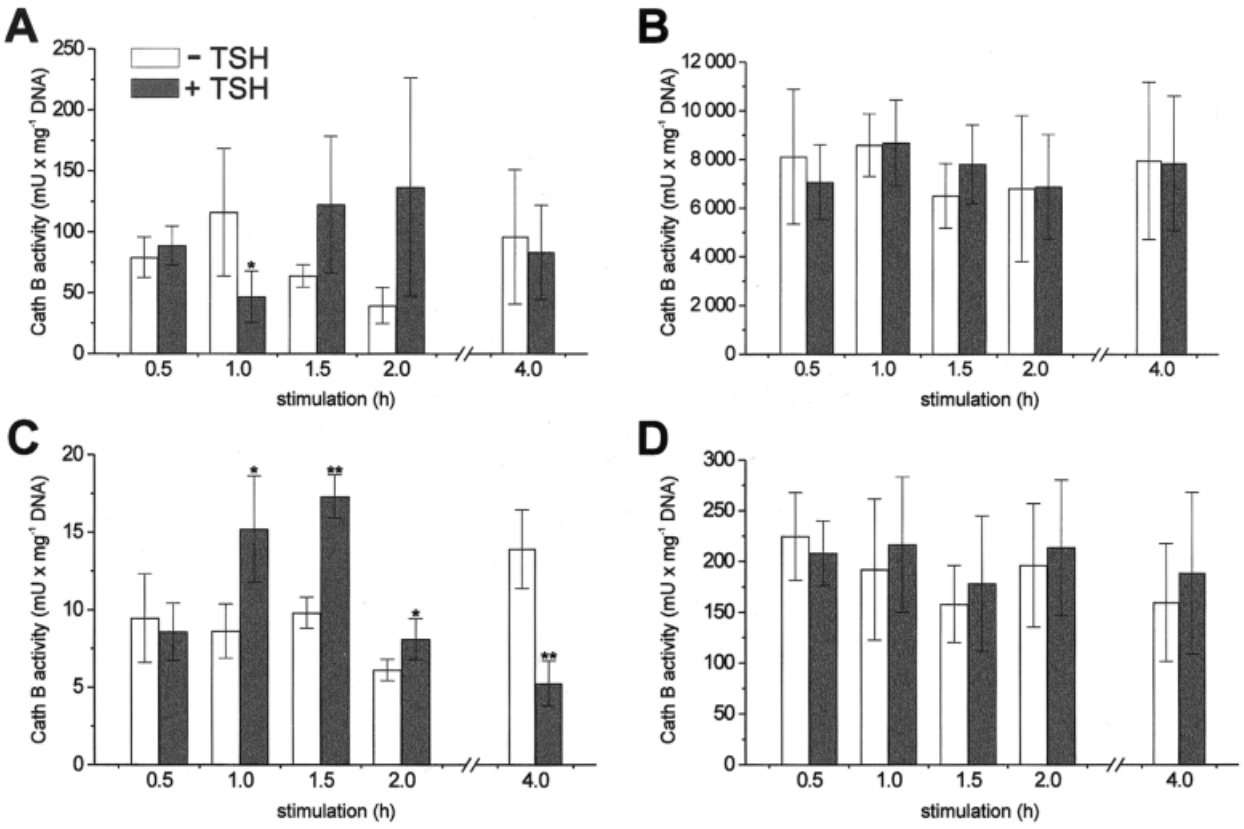

Fig. 6 Extracellular Cathepsin B Activity Is Upregulated by TSH.
Primary cultured porcine thyrocytes (A and B) or freshly isolated fragments from porcine thyroids (C and D) were incubated with culture medium without (white bars) or supplemented with 50 µU/ml TSH (grey bars) for the indicated time intervals. Plasma membrane preparations (A and C) or lysosomal fractions (B and D) were analyzed for cathepsin B activity at pH 7.2 (A and C) or pH 5.0 (B and D) by using a colorimetric assay.
Cathepsin B activities are expressed as mU per mg DNA and are given as mean ± standard deviation. Levels of significance are indicated as * for $p<0.05$ and as ** for $p<0.01$.

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gle-chain cathepsin B became increasingly detectable within the culture supernatants during 2–24 h of chase (Figure 5A, -TSH). These results indicated that mature forms of cathepsin B are constantly secreted from FRTL-5 cells (Figure 5B, white bars). However, when FRTL-5 cells were chased in TSH-containing stimulation medium, the amounts of extracellularly occurring single-chain cathepsin B were greater than those in media from non-stimulated cells (Figure 5A, compare +TSH with -TSH; Figure 5B, compare grey bars with white bars).

Hence, TSH stimulated the secretory release of mature forms of cathepsin B in both cellular systems studied. The results corroborate that TSH induces an enhancement of the extracellular proteolytic activity of thyroid epithelial cells within 2 h.

TSH Induces Upregulation of Proteolytically Active Cathepsin B in Plasma Membrane Fractions

Because TSH stimulation resulted in the extracellular accumulation of proteinases capable of releasing T_4 from Tg (see Figure 1), and because TSH induced the secretory release of mature forms of cathepsin B from intracellular stores (see Figures 4 and 5), we further analyzed whether TSH similarly affected the extracellular proteolytic cathepsin B activity of thyroid epithelial cells. For this purpose, primary cultured thyrocytes or freshly isolated thyroid fragments of porcine thyroids were incubated in culture medium supplemented without or with 50 µU/ml TSH for the indicated time intervals (Figure 6).

To test for pericellular *versus* intracellular proteolytic activity, cells or thyroid fragments were homogenized, and plasma membrane or lysosomal fractions were assayed for cathepsin B activity by incubation with Z-Arg-Arg-pNA in conditions reflecting the respective natural environment.

Plasma membrane preparations from non-stimulated cultured thyrocytes exhibited detectable cathepsin B activity at pH 7.2 (Figure 6A, white bars). After 1.5–2 h of TSH stimulation, the plasma membrane-associated cathepsin B activity was non-significantly increased by a factor of ~2.5 (Figure 6A, compare grey bars with white bars). In contrast, lysosomal cathepsin B activity at pH 5.0 was not altered by TSH stimulation of the cells (Figure 6B). However, the cathepsin B activity of lysosomal extracts exceeded that of plasma membrane fractions by a factor of about 80 (Figure 6A and B).

The subcellular distribution of enzymatically active cathepsin B was also studied in freshly isolated thyroid fragments (Figure 6C and D). Lysosomal cathepsin B activity was not altered by TSH stimulation of freshly isolated thyroid fragments (Figure 6D) and exceeded that of the plasma membrane fractions by a factor of approx. 20 (Figure 6C and D). Thus, exocytosis of mature cathepsin B seems to be more prominent in organ cultures than in cultured thyrocytes. Most importantly, TSH significantly upregulated the cathepsin B activity of plasma membrane fractions of thyroid fragments within 1–1.5 h by a factor of ~1.7 (Figure 6C). Hence, TSH does not alter the overall proteolytic activity of lysosomes, whereas TSH-induced upregulation of extracellular cathepsin B activity was observed in both model systems, *i.e*. in cultured thyrocytes and in freshly isolated thyroid fragments.

Discussion

Analysis of the biological significance of extracellular proteolytic activities is often limited to the immunohistochemical detection of the proteinases. Only few studies are available in which the proteolytic activity of extracellularly occurring enzymes has been evaluated (van Noorden *et al.*, 1987; Spiess *et al.*, 1994; Brix *et al.*, 1996; Chapman *et al.*, 1997; Bleeker *et al.*, 2000; Sameni *et al.*, 2000; Tepel *et al.*, 2000). However, because proteolytic cleavage is irreversible, even small proteolytic activities at certain locations might be of central importance for cell or tissue physiology. For the maintenance of thyroid function it is crucial that Tg is extracellularly solubilized from its covalently cross-linked storage form, because the large aggregates cannot be internalized as entirety by thyroid epithelial cells (Herzog *et al.*, 1992; Brix *et al.*, 2001). Here, we have analyzed the regulation of the secretory release of cathepsin B from thyroid epithelial cells, because it is known that cathepsin B is involved in the extracellular cleavage of Tg (Brix *et al.*, 1996, 2001). Our results suggest that TSH stimulates the secretion of mature forms of cathepsin B within 2 h. Furthermore, extracellular cathepsin B activity was upregulated by TSH, and the time course of TSH regulation of this activity closely matched that of Tg cleavage by extracellular means, *i.e*. of T_4 liberation mediated by extracellular Tg proteolysis. We therefore believe that even though only relatively small amounts of lysosomal enzymes occur extracellularly in a TSH regulated fashion, their proteolytic activity is of relevance for thyroid function. Because the assays for the detection of cathepsin B activity at extracellular locations were carried out at neutral pH, we conclude that even under such non-favorable conditions cysteine proteinases fulfill their task in the degradation of Tg.

Cellular Models for the Analysis of TSH Stimulation

In this study, we have included various cellular model systems to analyze TSH regulation of cysteine proteinase transport in thyroid epithelial cells, with the aim to compare the localization of cysteine proteinase activity with that of Tg proteolysis in a setup that mimicks the *in vivo* situation as closely as possible.

The normal concentration of TSH in the blood of newborns ranges from <12–20 µU/ml, with TSH levels exceeding 20 µU/ml being indicative of hypothyroidism (Shulkin and Utiger, 1985; Larsen, 1989; Magner, 1990). For the stimulation of primary cultured human thyrocytes, however, much higher concentrations of TSH are need-

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ed, *i.e*. 250 µU/ml TSH (Lamy *et al.*, 1990). After 4 days of TSH stimulation, increased proliferation and upregulation of the expression of various differentiation markers of thyroid epithelial cells has been observed in this model system (Lamy *et al.*, 1990). These results demonstrate the pleiotropic effects of TSH on thyroid epithelial cells with the pituitary hormone stimulating both, cell proliferation and cell differentiation (Herzog, 1984; Lamy *et al.*, 1990; Graebert *et al.*, 1997; Pietrzik *et al.*, 1998). In addition, TSH induced a strong retraction of human monolayer thyrocytes (Lamy *et al.*, 1990). Similarly, we have observed that TSH induced the formation of numerous cellular extensions, when FRTL-5 cells were grown under conditions of chronic TSH stimulation. FRTL-5 cells are most suitable as a model system, because this cell line displays a phenotype reminiscent of thyroid epithelial cells (Akamizu *et al.*, 1990). Furthermore, it has been described that desensitization to TSH occurs upon chronic TSH stimulation of porcine thyrocytes (Chambard *et al.*, 1990). This may be explained by the TSH induced downregulation of TSH receptors as it has been shown for FRTL-5 cells (Akamizu *et al.*, 1990). However, *in vivo* such chronic TSH stimulating conditions are indicative of severe dysfunctions of the thyroid gland leading to diseases such as hyperthyroidism and goiter formation (Larsen, 1989; Kohn *et al.*, 1993).

In healthy conditions, TSH levels in the blood are rapidly increased as a response to reduced thyroid hormone levels (Wollman, 1969; Ekholm, 1990). Thus, in order to mimick the conditions of acute TSH stimulation, TSH has to be administered very briefly and in concentrations which are tolerated by thyroid epithelial cells. Here, we have observed that stimulation of porcine thyrocytes or of FRTL-5 cells with ~50 µU/ml resulted in TSH induced effects without inducing dramatic changes in the cellular morphology. Similar amounts of TSH in the culture medium of porcine thyrocytes or of FRTL-5 cells are known to stimulate synthesis and secretion of Tg. However, these effects are part of a long-term response *via* the cAMP system, requiring several hours to days to reach a detectable level (Chambard *et al.*, 1990; Leer *et al.*, 1990). In contrast, induction of thyroid hormone liberation from the prohormone Tg and secretion of the former into the circulation are much faster events (Wollman, 1969; Ekholm, 1990; Kohrle, 2000).

Here, we were able to mimick the short-term TSH effects by incubation of porcine thyrocytes with 50 µU/ml TSH for few hours. In contrast to the proposal that thyroid hormone liberation occurs after endocytosis of Tg and its lysosomal breakdown (Herzog, 1984; Wollman, 1989; Ekholm, 1990; Rousset and Mornex, 1991; Molina *et al.*, 1996), we have observed that TSH stimulates Tg proteolysis by a strong increase in extracellular T_4 liberating activity. Because lysosomal Tg proteolysis was unaltered under conditions of acute TSH stimulation, we conclude that TSH specifically modulates the extracellular levels of thyrocyte proteinases. Biologically, such extracellular proteolytic activity is meaningful, since Tg, stored in the extracellular follicular lumen in covalently cross-linked form, needs to become solubilized from these luminal aggregates before endocytosis might occur (Brix *et al.*, 2001).

TSH Effects on Thyroidal Cysteine Proteinases

Since it is well established that cysteine proteinases are able to cleave Tg (Dunn and Dunn, 1982, 1988; Dunn *et al.*, 1991; Brix *et al.*, 1996), we thought to analyze cathepsin B as a prominent example of a thyroidal cysteine proteinase in more detail. It has been shown in rats that chronic TSH stimulation by subtotal thyroidectomy caused alterations in the lysosomal ultrastructure and in lysosomal enzyme activities (Krupp *et al.*, 1984). Treatment of FRTL-5 cells for up to 6 days with 25 – 150 mU/ml TSH resulted in a ~5-fold increase in the mRNA levels of cathepsin B (Phillips *et al.*, 1989). In rabbits, long-term TSH stimulation by a 24 h-treatment of the animals with 10 U bovine TSH caused an upregulation of cysteine proteinase activities within lysosomes (Dunn, 1984). More recently, in chronically hyperstimulated thyrocytes of patients suffering from Graves' disease, an increased immunoreactivity of cathepsin B has been observed (Shuja *et al.*, 1999). Hence, these studies have collectively shown that TSH upregulates cysteine proteinases, only, when administered for several days. In the pathological situation of Graves' disease this chronic TSH stimulation is caused by autoantibodies which permanently activate the TSH receptors.

In contrast, no alterations of cathepsin B mRNA levels were observed upon acute TSH stimulation of FRTL-5 cells (Petanceska and Devi, 1992). However, it is known that acute TSH stimulation *in situ* induces a movement of lysosomes from the basal to the apical pole of thyrocytes (De Robertis, 1941; Seljelid, 1967). Similarly, a redistribution of cathepsin B containing vesicles was observed in rat thyroids, and correlated with the diurnal changes of TSH levels in the blood of the animals (Uchiyama *et al.*, 1989). It was shown that at noon, when TSH levels are highest, vesicles containing cathepsin B occur at the apical pole of rat thyrocytes *in situ* (Uchiyama *et al.*, 1989). Hence, these studies suggested that acute TSH stimulation of the thyroid resulted in the lining up of lysosomes at the apical cell pole, *i.e*. underneath the apical plasma membrane facing the extracellular follicular lumen, in which Tg is stored. Here, we have observed a redistribution of cathepsin B containing vesicles from the perinuclear region to the cell periphery of FRTL-5 cells and of primary cultured porcine thyrocytes as a result of acute TSH stimulation. Both cellular systems were then shown to secrete mature forms of cathepsin B in a TSH dependent fashion, indicating that redistributed lysosomes might fuse with the plasma membrane as a response to acute TSH stimulation. In other cellular systems, *i.e*. fibroblasts or tumor cells, a redistribution of lysosomes similarly preceded the release of lysosomal enzymes into the pericellular space (Honn *et al.*, 1994; Rozhin *et al.*,

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1994; Sloane *et al.*, 1994; Ulbricht *et al.*, 1997; De Stefanis *et al.*, 1997; Calkins *et al.*, 1998; Martinez *et al.*, 2000). Fusion of lysosomes with the plasma membrane might be triggered by a rise in intracellular free Ca^{2+}-levels (Griffiths, 1996; Andrews, 2000). Indeed, it is known that TSH can activate the phosphatidylinositol cascade of thyrocytes, thereby inducing a rise in intracellular free Ca^{2+}-levels (Chiovato and Pinchera, 1991; Dumont *et al.*, 1992).

Our results suggest that TSH enhances the potency of thyroid epithelial cells to degrade Tg by extracellular proteolysis by promoting the fusion of peripheral lysosomes with the plasma membrane. This proposal is supported by our observation that cathepsin B activity in plasma membrane preparations of TSH stimulated cultured porcine thyrocytes or of freshly isolated porcine thyroid fragments was upregulated during the acute phase of the response. Therefore, we conclude that acute TSH stimulation is of biological significance in thyroid function as it regulates the transport and secretion of mature lysosomal cysteine proteinases. Enhanced extracellular levels of these enzymes appear to enable thyrocytes to degrade Tg in the follicular lumen of the thyroid.

Materials and Methods

Preparation of Thyroid Fragments

Porcine thyroid glands were obtained from the local slaughter house and transported on ice to the laboratory. Thyroid tissue was suspended and cut in Eagle's Minimum Essential Medium (MEM, BioWhittaker, Verviers, Belgium) into ~0.2 mm fragments using razor blades. After repeated washing, thyroid fragments were sedimented at 100 *g* for 75 s. Fragments were resuspended in 1 mg/ml collagenase in MEM and incubated for 30 min at 37 °C under 150-rpm agitation. The suspension was dissociated using siliconized glass pipettes with decreasing diameter from 1.0 – 0.6 mm. After filtration through 250 µm and 150 µm gauze, fragments were washed and collected in MEM without phenol red by repeated centrifugation at 100 *g* for 60 s. The resulting pellet was resuspended, and equal aliquots of thyroid fragments were pelleted by centrifugation at 300 *g* for 60 s prior to the stimulation experiment (see below). For cell culture, the resulting pellet was resuspended in culture medium.

Cell Culture

The pellet of isolated thyroid fragments was resuspended in MEM supplemented with 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.5 µg/ml amphotericin B, and 10% fetal calf serum (FCS). Cells were plated on cover glasses or tissue culture flasks, and incubated at 37 °C in 5% CO_2. For all experiments, cells were grown without any further passage to confluency which was reached 7 – 10 d after isolation.

FRTL-5 cells (Ambesi-Impiombato *et al.*, 1980) were purchased from the European Collection of Cell Cultures (ECACC, Salisbury, UK) and were grown at 37 °C and 5% CO_2 in F-12 Coon's modification (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) supplemented with 2.68 mg/ml sodium bicarbonate and 5% calf serum (CS). Antibiotics were added as listed above. Further additives to the culture medium of FRTL-5 cells (6H-medium) were 0.1 U/ml TSH, 0.166 mg/ml insulin, 0.362 µg/ml hydrocortisone, 2 µg/ml glycyl–histidyl-lysine, 1 µg/ml somatostatin, and 0.5 µg/ml transferrin (all from Sigma-Aldrich Chemie GmbH). For some experiments, FRTL-5 cells were grown in the above medium, but without the addition of TSH (5H-medium).

Immunolabeling after TSH Stimulation

FRTL-5 cells were grown on cover glasses in 6H-medium to approx. 70% confluency. Before stimulation, FRTL-5 cells were incubated with 5H-medium, *i.e.* culture medium without TSH, for 24 h. Stimulation was for 4 h at 37 °C and 5% CO_2 with 5H-medium without or with 3 µU/ml, 30 µU/ml, or 300 µU/ml TSH. As a control of continuous TSH stimulation, FRTL-5 cells incubated with 6H-medium which contained 0.1 U/ml TSH were used. Porcine thyrocytes grown on cover glasses were incubated with culture medium supplemented with or without 50 µU/ml TSH for 2 h at 37 °C and 5% CO_2.

After washing in phosphate buffered saline (PBS), cells were fixed with 8% paraformaldehyde in 200 mM Hepes (pH 7.4) for 30 min at room temperature. After washing in Hepes buffer, cells were permeabilized with 0.2% Triton X-100 in this buffer for 5 min at room temperature, followed by washing and subsequent blocking with 3% bovine serum albumin (BSA) in PBS. FRTL-5 cells were incubated with rabbit anti-rat cathepsin B antibodies (Upstate Biotechnology, distributed by Biozol, München, Germany) and secondary Cyanine 3 (Cy3)-conjugated goat anti-rabbit antibodies (Dianova, Hamburg, Germany). Porcine thyrocytes were incubated overnight at 4 °C with mouse anti-human mature cathepsin B antibodies (IM27; Calbiochem-Novabiochem GmbH, Bad Soden, Germany), and for 90 min at 37 °C with tetramethylrhodaminylisothiocyanate (TRITC)-conjugated secondary antibodies (Dianova).

Labeled cells were mounted on microscope slides in a mixture of 33% glycerol and 14% mowiol in 200 mM Tris (pH 8.5) supplemented with 5% 1,4-diazabicyclo(2.2.2)octane. Immunolabeled cells were viewed with an LSM 510 confocal laser scanning microscope (Zeiss, Oberkochen, Germany), and micrographs were stored in TIFF-format. Color coding was by Image Pro Plus 3.0.01.00 software (Media Cybernetics, L.P., Silver Springs, USA).

Biosynthetic Labeling and Immunoprecipitation of Secreted Cathepsin B

Primary cultured porcine thyrocytes were radiolabeled for 1 h or for 2 d with culture medium containing 40 µCi/ml [^{35}S]-methionine/[^{35}S]-cysteine (Amersham Pharmacia Biotech Ltd., Little Chalfont, UK). The non-radioactive chase medium was supplemented without or with 50 µU/ml TSH, and was applied for up to 48 or 4 h, respectively. Proteins of culture supernatants were precipitated with 0.5 mg/ml ammonium sulfate for 2 h at 4 °C prior to boiling for 10 min in extraction solution [0.125 M Tris-HCl (pH 6.8), 0.5% Triton X-100, and protease inhibitors]. Cells were lysed with extraction solution and cleared by centrifugation for 10 min at 14 000 *g* (4 °C). Iodoacetamide was added to cleared supernatants, which were used for immunoprecipitation with rabbit anti-human cathepsin B antibodies as described previously (Mach *et al.*, 1992). FRTL-5 cells were radiolabeled for 1 h in 5H-medium containing 30 µCi/ml [^{35}S]-methionine/[^{35}S]-cysteine. Chase was with 5H-medium supplemented with or without 50 µU/ml TSH for up to 24 h. Culture supernatants were cleared by centrifugation and lysosomal fractions were prepared as described below. Immunoprecipitation was with cleared supernatants and lysates of lysosomes by using rabbit anti-rat cathepsin B antibodies (Biozol), or rabbit anti-rat cathepsin B propeptide antibodies (kindly provided by Dr. John S. Mort, Montreal, Canada). Immunoprecipitates were collected and washed by protein A-coupled magnetic microbeads (Miltenyi

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Biotec, Bergisch Gladbach, Germany) according to the manufacturer's protocol. Before immunoprecipitation, culture supernatants, cellular extracts, or lysates of lysosomes were normalized to equal amounts of cells. Immunoprecipitates were analyzed by SDS-gels.

SDS-Gels, Autoradiography and Densitometric Quantitation of Gels

Immunoprecipitates were boiled in sample buffer (10 mM Tris-HCl, pH 7.6, 0.5% SDS, 25 mM DTT, 10% glycerol, 25 µg/ml bromophenol blue). Samples were separated by SDS-polyacrylamide gel electrophoresis (PAGE; Laemmli, 1970), followed by autoradiography. Autoradiographs were evaluated densitometrically by OptiQuant version 3.0 (Packard Instrument Co., Meriden, CT, USA).

Subcellular Fractionation after TSH Stimulation

Freshly isolated thyroid fragments or cultured porcine thyrocytes were incubated with stimulation medium for time intervals of 0.5, 1.0, 1.5, 2.0, or 4.0 h at 37 °C under agitation. MEM without phenol red containing 50 µU/ml TSH was used as stimulation medium. Controls were incubated with the same medium, but without TSH. After incubation, thyroid fragments were pelleted (300 *g*, 60 s), and resuspended in 0.1 M Soerensen phosphate buffer (KH_2PO_4 and Na_2HPO_4, pH 7.2) supplemented with 0.25 M sucrose and 5 mM EDTA (homogenization buffer). Cultured thyrocytes were incubated with stimulation medium as described above, and were removed from tissue culture flasks by using rubber policemen. Cells were resuspended in homogenization buffer.

The suspensions of thyroid fragments or thyrocytes were homogenized on ice using a Dounce homogenizer (Kontes Co., Vineland, USA). Nuclei were removed by centrifugation for 5 min at 900 *g* and 4 °C. Nuclear fractions were analyzed for their DNA content to estimate cell numbers (see below). The supernatants were centrifuged for 10 min at 10 000 *g* and at 4 °C to collect lysosomes. The resulting pellet was resuspended in PBS (pH 5.0) supplemented with 0.2% Triton X-100, and incubated on ice for 30 min. The supernatants of the final centrifugation step for 10 min at 15 000 *g* (4 °C) were used as lysosomal fractions for the cathepsin B activity assays and for the thyroid hormone liberating assay. Plasma membrane vesicles were isolated from the supernatants of the 10 000 *g* centrifugation step by overlaying onto cushions of 1.2 M sucrose and 0.32 M sucrose in Soerensen phosphate buffer with EDTA, and centrifugation for 120 min at 100 000 *g* (4 °C). The band at the interphase of 0.32 and 1.2 M sucrose was removed with a syringe, resuspended in PBS, and pelleted for 60 min by centrifugation at 100 000 *g* (4 °C). The pellet contained plasma membrane vesicles which were lysed for 30 min on ice with PBS (pH 7.2) supplemented with 0.2% Triton X-100. Cleared supernatants (see above) were used for the cathepsin B activity assays. The purity of the individual subcellular fractions was assessed by electron microscopy as described previously (Brix *et al.*, 1996).

DNA Determination

The DNA contents of pellets of nuclear fractions were determined according to Burton (1956). Samples were first incubated with ice-cold 0.5 N perchloric acid (PCA) for 60 min. Precipitated DNA was collected and washed by repeated centrifugation for 5 min at 10 000 *g* (4 °C). Hydrolysis of DNA was for 20 min at 75 °C, and cleared supernatants were mixed with an equal volume of color reagent (665 mg diphenylamine, 670 µl concentrated sulfuric acid, 170 µl of 0.33 mg/ml acetaldehyde in 24.2 ml concentrated acetic acid) for 16 – 20 h at room temperature. Samples were again cleared by centrifugation and the absorption at 600 nm was determined photometrically. Calibration curves were prepared with calf thymus DNA (Sigma-Aldrich Chemie GmbH). One µg of DNA was equivalent to 10^6 cells (Thomas and Farquhar, 1978).

In vitro Degradation of Tg and Radioimmunoassay

Culture supernatants or lysosomal fractions of TSH stimulated porcine thyrocytes were incubated after reactivation of cysteine proteinases by L-cysteine incubation with Tg for 30 min at 37 °C at pH 7.2 or pH 5.0, respectively (Brix *et al.*, 1996; Tepel *et al.*, 2000). The amounts of liberated T_3 or T_4 were quantitated by radioimmunoassay (Brahms Diagnostica GmbH, Berlin, Germany). Zero time controls were subtracted from values obtained after 30 min of incubation with Tg. They were normalized to equal amounts of cells.

Cathepsin B Activity Assays

The activity of cathepsin B was determined by a colorimetric assay according to Barrett (1972), and Barrett and Kirschke (1981) with modifications. Lysates of lysosomal or plasma membrane fractions were diluted in enzyme dilution buffer [0.01% BSA, 1.47 mM Na_2EDTA in 100 mM phosphate buffer (Na_2EDTA/PP) adjusted to pH 5.0 or pH 7.2, respectively]. Fractions were reactivated for 5 min at 40 °C by the addition of L-cysteine in Na_2EDTA/PP to a final concentration of 2 mM. The substrate N-benzyloxycarbonyl-arginyl-arginine-p-nitroanilide (Z-Arg-Arg-pNA; Bachem Biochemica GmbH, Heidelberg, Germany) was added to a final concentration of 0.125 mM. Enzyme dilution buffer was used as a blank. Incubations were for 60 min at 40 °C. The reactions were stopped by the addition of 2 M Tris-HCl (pH 9.0), and the absorption at 410 nm was determined photometrically. Calibration curves were prepared by using bovine spleen cathepsin B (Sigma-Aldrich Chemie GmbH) as a standard.

Statistic Evaluations

All statistics were done using standard computer software and levels of significance were determined by one-way ANOVA (Origin, MicroCal Software, Northampton, MA, USA).

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