BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **234,** 133–136 (1997)
ARTICLE NO. RC976601

Perturbation of Cellular Calcium Delays the Secretion and Alters the Glycosylation of Thyroglobulin in FRTL-5 Cells

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Received March 21, 1997

Treatment of FRTL-5 cells with a Ca^{2+} ionophore, A23187, or a specific inhibitor of the endoplasmic reticulum Ca^{2+}ATPases, thapsigargin, delayed thyroglobulin secretion. The secreted thyroglobulin showed an increased electrophoretic mobility and a reduced sensitivity to neuraminidase. Only thyroglobulin that was still in the endoplasmic reticulum was sensitive to the Ca^{2+}-perturbant drugs as shown by experiments in which the drugs were added at different times during a chase. Analysis of the carbohydrate chains by BioGel P4 showed that thyroglobulin secreted in the presence of the Ca^{2+}-perturbants displayed an increased ratio high mannose/complex chains. © 1997 Academic Press

Thyroglobulin (Tg) is the major secretory protein of the thyroid gland and the molecular site of synthesis of thyroid hormones. Tg is synthesized and cotranslationally glycosylated in the rough ER by the transfer of $\geqslant$20 $Glc_3Man_9GlcNac_2$ oligosaccharides (high mannose chains). The complex chains are built in the Golgi complex. Finally, Tg is exocytosed into the follicular lumen (1).

The ER lumen is a major store of cellular calcium, the concentration being between 1.8 and 5.4 mM (2). The role of ER Ca^{2+} levels on protein transport has been recently studied (3). However nothing is known on the effect of ER Ca^{2+} on the transport and processing of Tg. We reasoned that this question is of importance given the extremely large size of Tg (660 kDa) and its structural complexity ($\geqslant$20 oligosaccharides chains and $\geqslant$60 disulphide bonds). In addition, Tg binds 68 Ca^{2+} ions per dimer that modulate its conformation (4). Thus, the effects of Ca^{2+} on Tg could be amplified respect to other proteins and therefore better understood. We show that treatment of FRTL-5 cells either with the Ca^{2+} ionophore A23187 or the inhibitor of the ER Ca^{2+}-ATPases, thaps, delays the secretion of newly synthesized Tg. Moreover, the secreted Tg shows an altered glycosylation, consisting in an inhibition of the processing of the high mannose chains and of the addition of sialic acid on the complex chains. Such alterations of glycosylation have not been described for other proteins following a Ca^{2+} depletion in the ER lumen.

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Abbreviations: Tg, thyroglobulin; thaps, thapsigargin; ER, endoplasmic reticulum.

MATERIALS AND METHODS

Materials. Insulin and Pansorbin were from Calbiochem, [^{35}S]-Methionine (1000Ci/mmol) from Amersham, EndoH and neuraminidase from Boehringer Mannheim, all other chemicals from Sigma.

Cell culture. FRTL-5 cells (ATCC CRL 8305) were grown as previously described (5).

Protein labelling. Cells were incubated for 1 h in methionine-free medium, pulse-labelled for 5 min in the same medium plus 50 μCi/ml [^{35}S]-methionine and chased with an excess of cold methionine (1000-fold) with or without 5 μM A23187 or 5 μM thaps. After various chase times, medium was collected, cells lysed as previously described (5).

Carbohydrate labelling. Cells were pulse-labelled for 15 min with 20 μCi/ml [^{3}H]mannose and one-tenth the usual concentration of glucose and chased with an excess of cold mannose (1000 fold) with or without 5 μM A23187 or 5 μM thaps. After 3 hours, medium was collected and Tg immunoprecipitated.

Immunoprecipitation and SDS-PAGE. Tg was immunoprecipitated as previously described (5). Samples were dissolved in 0.625 M Tris pH 6.8, 2% SDS, 0.35% β-mercaptoethanol, 20 mM dithiothreitol, 10% glycerol, heated to 95°C for 5 min, and loaded on 5% SDS-gels.

Pronase digestion and chromatography. Pronase digestion of secreted immunoprecipitated Tg was described (5). Chromatography of the pronase digest used a Bio-Gel P4 column (200-400 mesh) which was 150×0.7 cm. The column was in 0.1 M Tris-hydrochloride, pH 8.25. Elution volumes were 0.2 ml, the flow rate was 3 ml/h. The high mannose glycopeptides standards were prepared as described (6).

Other procedures. EndoH, and neuraminidase digestions were previously reported (5).

0006-291X/97 $25.00
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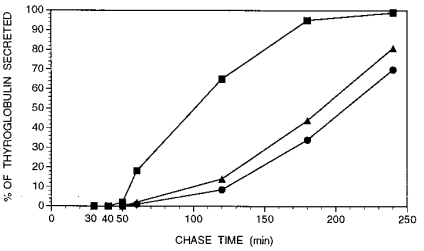

FIG. 1. Thaps and A23187 inhibit Tg secretion in FRTL-5 cells. Cells were chased without (■) or with either thaps (●) or A23187 (▲) for the times indicated. Secreted and intracellular Tg were immunoprecipitated and resolved by SDS-PAGE. The percentage of newly synthesized secreted Tg is plotted against the chase time.

RESULTS

A23187 and thaps Inhibit Secretion and Alter the Glycosylation of Newly Synthesized Tg

The newly synthesized Tg began to be secreted into the medium 60 min after synthesis. Addition of either 5 μM thaps or A23187 at the beginning of the chase slowed down Tg secretion (fig. 1). The total Tg (secreted plus intracellular) from cells incubated with or without

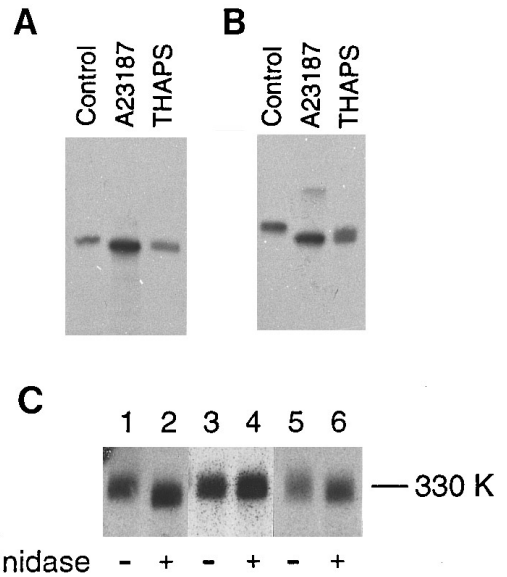

FIG. 2. The Tg secreted by FRTL-5 cells in the presence of A23187 and thaps shows an increased electrophoretic mobility and a reduced sensitivity to neuraminidase. A and B: Cells were chased without (control) or with thaps or A23187 for 180 min. Secreted Tg was immunoprecipitated and resolved by reducing (A) or non-reducing (B) SDS-PAGE. An attempt had been made to immunoprecipitate equal amounts of Tg by taking different amounts of medium. C: Cells were chased without (lanes 1-2) or with A23187 (lanes 3-4) or thaps (lanes 5-6) for 180 min. Secreted Tg was mock-digested or digested with neuraminidase. Different exposure times were used for lines 1-2, 3-4, 5-6, thus the faster mobility of Tg with the drugs is not shown.

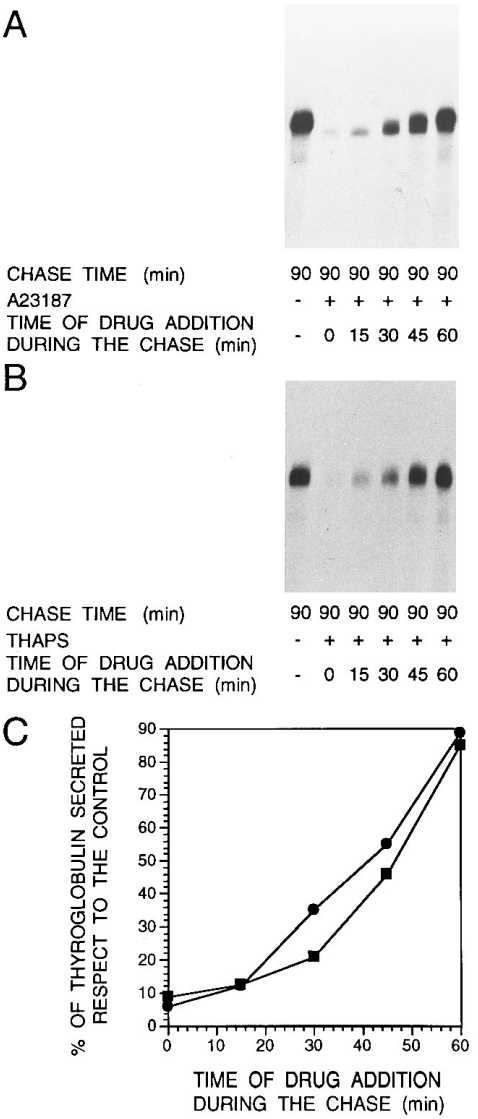

FIG. 3. The inhibition of Tg secretion by A23187 and thaps is relieved by delaying drug addition. Cells were chased for 90 min. During the chase A23187 (A) or thaps (B) were either not added (lanes 1, A and B) or added at the beginning of the chase (time of drug addition 0, A and B) or added at different times (time of drug addition 15, 30, 45, 60 min, A and B). Secreted (shown in A and B), and intracellular Tg (not shown) were immunoprecipitated and resolved by SDS-gel. The percentage of Tg secreted with the drugs respect to that secreted in controls is plotted against the time of drug addition (C).

the Ca^{2+}perturbants was constant (not shown), ruling out Tg intracellular degradation.

In addition to the quantitative effect on Tg secretion (fig. 1), the drugs increased also the migration of the secreted Tg on reducing and non-reducing SDS-gels (fig. 2A and 2B). Since this effect could be caused by an altered glycosylation, we subjected the secreted Tg to neuraminidase digestion. Tg secreted in the presence of the drugs displayed a smaller shift (thaps) or no

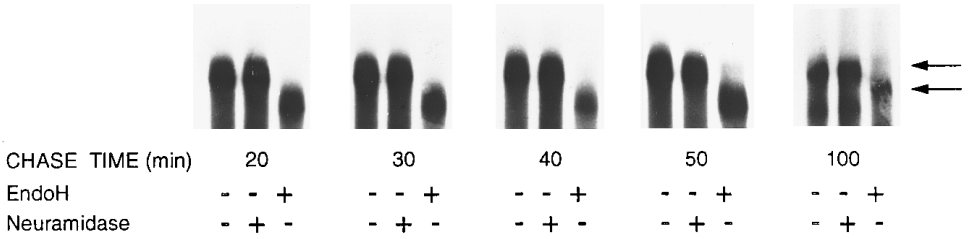

FIG. 4. Time-course of the acquisition of endoH-resistance and neuraminidase-sensitivity by Tg. Cells were chased without the drugs, and lysed at different times of chase. Medium from each plate was divided in three aliquots, immunoprecipitated, and mock-digested or digested with endoH or neuraminidase.The samples were resolved by SDS-PAGE.

shift (A23187) after neuraminidase digestion (fig. 2C, compare the shift between lanes 1 and 2 with the shifts between lanes 3 and 4 or 5 and 6), indicating that A23187 and thaps inhibited the sialic acid addition on the complex chains.

The Inhibition of Tg Secretion by A23187 and thaps Is Relieved by Delaying Drug Addition During the Chase

In the previous experiments A23187 and thaps were added to the cells immediately after the period of protein synthesis (pulse). However, it was interesting to check how soon after synthesis the secretion of Tg became resistant to A23187 and thaps. Cells were pulse-labelled and chased for 90 min. The drugs were either not added (fig. 3, A and B, lane 1) or added immediately after the pulse (fig. 3, A and B, lane 2) or added 15, 30, 45, and 60 min after the pulse (fig. 3, A and B, lanes 3-6). Secreted Tg was immunoprecipitated and resolved on reducing SDS-gels. The inhibition of Tg secretion progressively disappeared by delaying the addition of the drugs (fig. 3, A, B, and C). Also, the alterations of the electrophoretic mobility of Tg showed a progressive disappearance. Therefore, moving along the exocytic pathway, Tg became insensitive to A23187 and thaps 60 min after synthesis. To identify the organelle in which the resistance to the drugs was acquired, we measured the transit time of Tg at the medial- and trans-Golgi by studying in control cultures the time of acquisition of endoH-resistance and neuraminidase-sensitivity (7). Cells were pulse-labelled for 5 min and chased for various times. Intracellular Tg was immunoprecipitated and digested with endoH and neuraminidase. It took about 50 min to the first Tg molecules to show endoH-resistance, i. e., to reach the medial-Golgi (fig. 4, arrows indicate the positions of endoH-resistant and sensitive Tg). Since 90% of the Tg molecules became resistant to the Ca^{2+}-perturbant drugs 60 min after synthesis (fig. 3, panel C), the resistance preceded the arrival at the medial-Golgi and, very likely, was acquired when Tg was still in the ER.

Analysis of the Carbohydrate Chains of Tg Secreted in the Presence of A23187 and thaps

The glycosylation of Tg was studied by labelling cells with ^{3}H-mannose. Secreted Tg was immunoprecipitated, digested with pronase and chromatographed on a BioGel P4 column. As shown in fig. 5 in control conditions the glycopeptides obtained from Tg separated in two major peaks. The first one most likely represented glycopeptides with complex chains since it was selectively labelled with ^{3}H-galactose. The second peak represented glycopeptides with high mannose chains. With thaps the high mannose peak increased respect to the complex peak. With A23187 the predominant

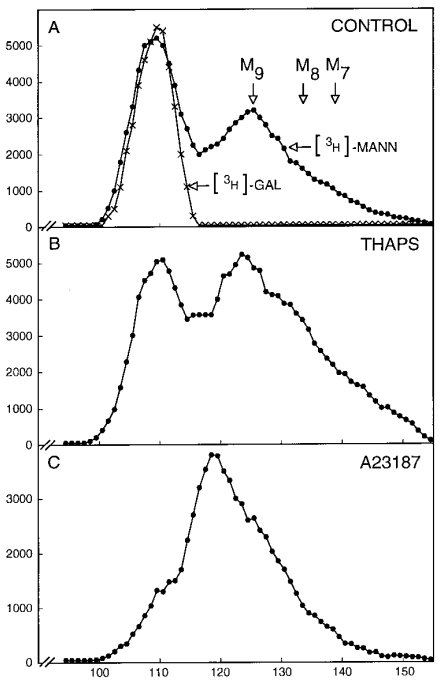

FIG. 5. Pronase-digested Tg secreted with A23187 and thaps shows altered profiles on BioGel P4. Cells were chased without (A) or with either thaps (B) or A23187 (C) for 180 min. Secreted Tg was immunoprecipitated and digested with pronase. After desalting the material was chromatographed on a Bio Gel P4 column. The recoveries were greater than 95%.

peak eluted at an intermediate position between the peaks present in the controls, that were reduced to two shoulders.

DISCUSSION

In this study it is shown that both the secretion and the glycosylation of Tg are altered when FRTL-5 cells are treated with a Ca^{2+} ionophore, A23187, and an inhibitor of the ER Ca^{2+}ATPases, thaps. We have used thaps to unambiguously study the effect of depleting the Ca^{2+} stores of the ER. Thus, thaps specifically inhibits the ER Ca^{2+} dependent ATPases (8). Accordingly with their action on the ER Ca^{2+} stores, A23187 and thaps inhibit Tg secretion and alter Tg glycosylation only if added to cells before Tg arrival at the medial-Golgi so likely when Tg is still in the ER.

The delay of Tg secretion caused by A23187 and thaps is probably linked to an alteration in the folding as has been postulated for other proteins (4). Tg binds as much as 68 Ca^{2+} ions per dimer that modulate its conformation (5). The Ca^{2+} requirement to achieve a correct conformation and its structural complexity (660 kDa, $\geqslant$20 oligosaccharides chains and $\geqslant$60 disulphide bonds) make the effects of Ca^{2+}depletion on Tg particularly pronounced. In fact, the alterations of glycosylation described here have not been described for other proteins.

The glycosylation of Tg is a regulated process. TSH is up to now the major known stimulator (9-12). In the normal thyroid hypoglycosylated variants of Tg exist (13, 14), although the mechanism underlying their production remains unknown. In this study such a mechanism is perhaps uncovered. In response to a loss of Ca^{2+} from the ER lumen, the conversion of high mannose chains to complex chains and the addition of peripheral sialic acid is inhibited. It would be interesting to investigate if these opposite effects on Tg carbohydrate moiety have some consequences on thyroid hormonogenesis.

ACKNOWLEDGMENTS

We thank Professors Gaetano Salvatore and Eduardo Consiglio for support.

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