Intracellular Distribution and Regulation of Calpains in the Thyroid Gland

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We studied the distribution of calpains in various subcellular thyrocyte fractions and evaluated the possibility for direct activation of calpain localized in the plasma membrane by thyrotropin. Direct activation of calpain bound to the plasma membrane did not underlie transduction of the thyrotropin signal to Ca^{2+}-dependent proteinases.

Key Words: *thyroid gland; thyrotropin; proteolysis; calpains; regulation*

Activation of intracellular Ca^{2+}-dependent proteinases under physiological conditions and the mechanisms underlying functioning of calpains in the presence of excess endogenous inhibitor calpastatin [6] and the absence of micro- and millimolar concentrations of Ca^{2+} necessary for their activation remain unclear [6, 7]. Our previous studies demonstrated rapid thyrotropin-induced activation of calpains in thyrocytes independently on Ca^{2+} content in the cytoplasm [1,2]. Compartmentalization of calpain and calpastatin is probably the factor responsible for regulation of calpains.

Here we studied the distribution of calpains in various subcellular fractions of the thyroid gland (TG) and evaluated the possibility for direct activation of calpain localized in the plasma membrane by thyrotropin.

MATERIALS AND METHODS

Experiments were performed on 80 male outbred albino rats. TG (2-4 per sample) was homogenized (1:5) in a medium containing 50 mM Tris, 4 mM ethylenediaminetetraacetic acid (EDTA), and 140 mM NaCl (pH 7.6, ratio 1:5) and centrifuged at 1000*g* for 10 min. The precipitate was resuspended in the same medium, and the supernatant was centrifuged at 20,000 and 105,000*g* for 30 and 120 min, respectively. The precipitates obtained after centrifugation were resuspended and used to measure calpain activity (CA). CA in the final supernatant was estimated after removal of endogenous inhibitors by the method of acid fractionation [8] or spectrophotometrically by the rate of alkali-denatured casein hydrolysis [8]. The plasma membrane-enriched fraction was obtained by the method [4] with modifications. TG homogenate in Tris buffer containing $NaHCO_3$ was centrifuged at 500 and 1500*g* for 10 and 15 min, respectively (membrane enzymes 5'-nucleotidase and leucyl aminopeptidase served as the markers [4]). The precipitate obtained after the 2nd centrifugation was washed 2 times and resuspended in a medium containing 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM Na_2HPO_4, and 50 mM Tris. CA in the obtained fraction was monitored by hydrolysis of fluorogenic substrate N-Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin (BAPTA) [3] in the presence or absence of 1 mM Ca^{2+}.

Ca^{2+}-free medium was obtained by adding chelator BAPTA (final concentration 3 mM) to the buffer. Protein content was measured by the method of Bradford. Thyrotropin was added after 6-min incubation with the substrate (immediately after the 1st measurement of activity).

Bovine thyrotropin (TSH), N-acetyl-Leu-Leu-norleucinal (calpain I inhibitor), N-acetyl-Leu-Leu-methioninal (calpain II inhibitor, Sigma), Tris, EDTA (Reanal), and Hammarsten's casein (Merk) were used. The results were analyzed by Student's *t* tests.

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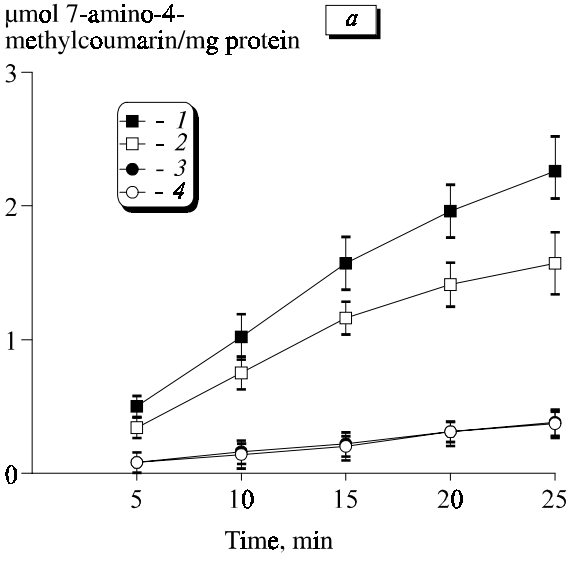
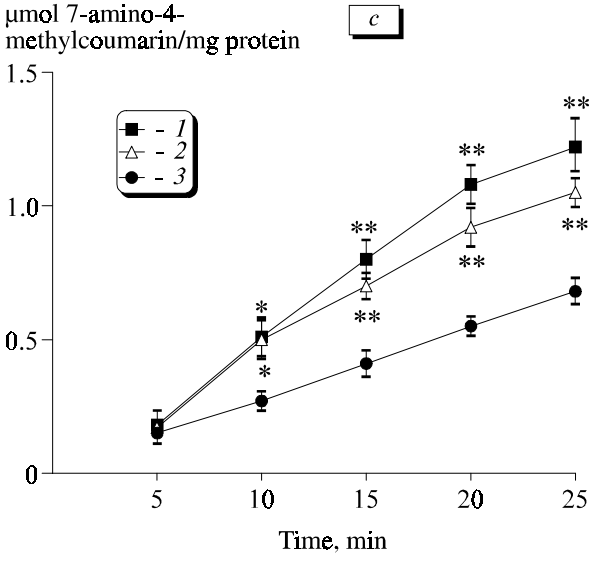
RESULTS

CA was detected in the microsomal (105,000*g*), mitochondrial (20,000*g*), and nuclear (1000*g*) fractions: 392.43±43.45, 17.95±2.51, and 8.88±2.49 nmol tyrosine/mg protein/h, respectively. In the cytosol, CA was found only after isolation of calpain from the endogenous inhibitor (725.18±86.34 nmol tyrosine/mg protein/h). Thus, TG cells have active (associated with intracellular membranes) and inactive (cytosolic) calpains. Our results are consistent with published data [5] on different subcellular localization of calpain and calpastatin in porcine TG cells revealed by immunofluorescence labeling.

Incubation with fluorogenic calpain-specific substrate N-Suc-Leu-Leu-Val-Tyr-7-amino-4-methylcoumarin [3] showed the presence of Ca^{2+}-dependent and Ca^{2+}-independent proteolytic activities in TG fractions enriched with plasma membranes (Fig. 1). Highly specific synthetic calpain I and II inhibitors [3] suppressed not only Ca^{2+}-dependent, but also Ca^{2+}-independent activity (Fig. 1). Therefore, the obtained fraction contained not only calpains, but also Ca^{2+}-independent proteinases differing in the sensitivity to inhibitors and possessing the ability to cleave the substrate (similar to calpains). The type of these enzymes requires further studies.

Thyrotropin in physiological concentrations of 10^{-3} and 10^{-2} U/ml had no effect on proteolytic activity in the fraction enriched with plasma membranes (data not shown). Increasing the concentration of thyrotropin to 10^{-1} and 50×10^{-1} U/ml slightly decreased Ca^{2+}-independent activity (Fig. 1), did not modulate the rate of substrate hydrolysis in the presence of Ca^{2+}, and increased Ca^{2+}-dependent activity. Thus, thyrotropin directly modulates proteinase activity in TG plas-





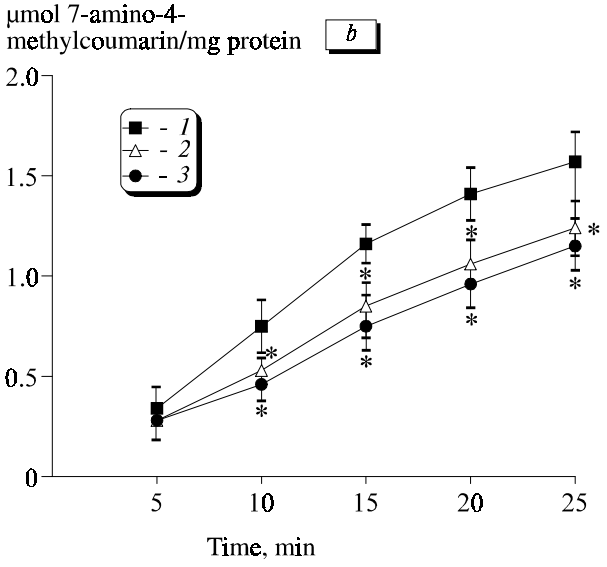


Fig. 1. Proteolytic activity in plasma membrane-enriched thyroid gland fraction (*a*) and thyrotropin-induced changes in Ca^{2+}-independent (*b*) and Ca^{2+}-dependent activities (*c*): *a*) in the presence of 1 mM Ca^{2+} (*1*), in Ca^{2+}-free medium (*2*), in the presence of 1 mM Ca^{2+} and calpain I inhibitor (*3*), and in Ca^{2+}-free medium with 1 mM Ca^{2+} and calpain II inhibitor (*4*); *b*, *c*) control (without thyrotropin, *1*); and in the presence of 10^{-1} (*2*) and 5×10^{-1} U/ml thyrotropin (*3*). *$p<0.05$ and **$p<0.01$ compared to the control.

ma membranes and changes the ratio between Ca^{2+}-dependent and Ca^{2+}-independent enzymes. This effect produced by thyrotropin in high concentrations was less pronounced than activation of TG calpains after thyrotropin addition to thyrocyte suspension [1] or *in vivo* administration of this hormone [2].

Thus, direct activation of plasma membrane-bound calpain is not the major mechanism underlying transduction of thyrotropin signals to Ca^{2+}-dependent proteinases. The presence of calpains not masked by calpastatin and associated with intracellular membranes suggests that proteinase activity is modulated via changes in their intracellular localization. This mechanism of thyrotropin signal transduction requires detailed investigations.

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