Tissue Calmodulin Levels in Normal and Graves’ Thyroids

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Abstract

Calmodulin levels in normal human thyroids and Graves’ disease thyroids were measured by specific radioimmunoassay in the presence of ethyleneglycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA). The calmodulin levels in tissues from patients with Graves’ disease treated with thionamide drugs were significantly higher than those in normal tissues from euthyroid patients with solitary cold nodules (normal: 484±50 ng/mg protein, mean±SE, n=15; Graves’: 901±54 ng/mg protein, n=48, p<0.001). Such a rise in calmodulin levels in Graves’ disease thyroids was also present even after the administration of 50 ng of T3 for 5 days before operation (828±137 ng/mg protein, n=6, p<0.01).

Calmodulin levels in Graves’ disease thyroids were closely related to the cell height of follicular epithelium. Calmodulin levels in a columnar cell predominant group were significantly higher than those in a flat cell predominant or a cuboidal cell predominant group (columnar cell predominant: 1150±118 ng/mg protein, n=13; flat cell predominant: 561±125 ng/mg protein, n=3, p<0.05; cuboidal cell predominant: 596±40 ng/mg protein, n=25, p<0.001).

The increase in calmodulin content in Graves’ disease thyroid could therefore possibly be attributed to the stimulation of the thyroid gland by the thyroid stimulating antibody. An immunofluorescence study demonstrated the presence of calmodulin immunoreactivity in the thyroid epithelial cells, particularly enriched in the apical border in the form of a granulated structure.

It is established that Ca++ seems to have an informational role in many tissues (Rasmussen, 1970; Rasmussen and Goodman, 1975; Berridge, 1975; Rebhun, 1977) and also in the thyroid, as well as cyclic AMP. Recent evidence has indicated that calmodulin acts as an intracellular Ca++ receptor and mediates the Ca++ regulation of an extensive range of fundamental cellular activities, including cyclic nucleotide and glycogen metabolism, protein phosphorylation, microtubule assembly and disassembly,

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Ca++ flux and the activities of NAD kinase, tryptophan-5'-monooxidase and phospholipase A_2 (Means and Dedman, 1980; Wang and Waisman, 1979; Cheung, 1980). The fact that calmodulin regulates so many physiological processes indicates the possibility that alteration of the intracellular level of this protein could also be one of the important mechanisms in control of responses mediated through Ca++. In fact, intracellular calmodulin levels are reported to be altered in a variety of conditions, such as fat cell and liver cell in diabetes (Solomon et al., 1979; Smoake and Solomon 1980; Morley et al., 1982), interferon treated human WISH cell (Bourgeade et al., 1983) and human red and white cells in chronic renal failure (Mooreadian et al., 1983). As do other tissues, human and bovine thyroids contain calmodulin and calmodulin dependent enzymes (Nagasaka and Hidaka 1976; Yagura et al., 1978; Kasai and Field, 1982; Kobayashi et al., 1979). In Graves' disease thyroid, phosphodiesterase activity, which is supposed to be stimulated by calmodulin, was reported to be significantly increased, as compared to that in normal human thyroid (Nagasaka and Hidaka, 1976). Therefore, we consider it important to determine the level of this protein in Graves' disease and normal human thyroids by means of specific radioimmunoassay in the presence of EGTA. Furthermore, localization of calmodulin in normal human thyroid tissue was determined by means of an indirect immunofluorescence microscopy procedure.

Materials and Methods

Thyroid tissues

Graves' disease thyroid tissues were obtained from 48 patients with Graves' disease when they underwent subtotal thyroidectomy after an appropriate period of antithyroid drug therapy. In these patients, serum T_3, T_4 and TSH levels determined at the time of operation were within their respective normal ranges. The diagnosis of Graves' disease had been made previously on the basis of the history of symptoms and signs of hyperthyroidism with diffuse goiter and elevated serum T_3 and T_4 levels and thyroidal 131I uptake. The diagnosis was also confirmed histologically after operation. Six patients with Graves' disease were administered T_3 (50 µg/day) for 5 days together with an antithyroid drug just before operation, and their serum TSH levels at the time of operation were suppressed to 2.5±0.5 µU/ml (mean±SE). Normal human thyroid tissues were obtained from 15 euthyroid patients with solitary cold nodules. Tissues adjacent to single nodules were found to be normal histologically and used as normal thyroid tissues in this study.

These tissues obtained at the time of operation were stored at −20°C until use for determination of the calmodulin level.

Calmodulin levels in excised thyroid tissues

Thyroid tissues were weighed, thawed and then homogenized with all-glass homogenizers in 20 volumes of a medium consisting of 125 mM borate buffer, pH 8.4, 1 mM EGTA and 75 mM NaCl. Aliquots were then removed for protein determination by the Lowry method (Lowry, Rosebrough, Farr and Randall 1951). The homogenates were heat-treated at 90°C for 5 min and rapidly cooled to 4°C by immersion into a methanol: dry ice bath. The heat-treated samples were then centrifuged at 10,000 g for 30 min and the supernatants were used for the assay. Calmodulin levels were measured in duplicate by RIA using CAABCO calmodulin kits (CAABCO, Houston, Texas). All samples were determined in the same assay run. Intra assay precision, determined on three different samples, was 96.5±13.0 ng/mg, 13.4% (mean±SD and CV, n=6) 173.6±16.7 ng/ml, 9.6% (n=7) and 253.7±32.1 ng/ml, 12.7% (n=6). Calmodulin levels were expressed as ng of calmodulin per mg protein.

Histological examination of Graves' disease thyroid

The Graves' disease thyroid tissues were investigated histologically by a pathologist and divided into a columnar cell predominant, a cuboidal cell predominant or a flat cell predominant group on the basis of the height and shape of the principal thyroid epithelial cells.
Immunofluorescence study

One specimen from normal thyroid tissue was snap frozen and stored at $-80^\circ C$ and 4 mm cryostat sections were prepared at $-20^\circ C$. Sections were fixed with 95% ethanol, reacted with sheep anticalmodulin antibody (CAABCO, Houston, Texas), washed again with phosphate-buffered saline, and then reacted with fluoresceinisothiocyanate (FITC)-conjugated antisheep IgG antibody (Cappel Laboratories, Cochranville, PA). After complete washing, the localization of fluorescence was examined with a fluorescent microscope. As a methodological control, thyroid sections were reacted with FITC-conjugated antisheep IgG antibody in the presence of normal sheep IgG instead of anticalmodulin IgG.

Statistical analysis

Statistical analysis of the significance of difference between groups was carried out by Student’s t-test.

Results

As shown in Fig. 1, the mean value with SE in 15 normal tissues was $484 \pm 50$ ng/mg protein. The normal range calculated as the mean $\pm 2SD$ from these normal values was from 101 to 867 ng/mg protein. The values in 48 Graves’ disease thyroids varied broadly from 176 to 1692 ng/mg protein with the mean and SE of $901 \pm 54$ ng/mg protein. The calmodulin levels in Graves’ disease thyroids were significantly higher than those in normal thyroids ($p<0.001$) and 16 out of 48 patients with Graves’ disease (33.3%) showed values higher than the normal range. Furthermore, a significant increase ($p<0.01$) in the calmodulin levels in Graves’ disease thyroids was also seen even after the administration of daily dose of 50 µg of T₃ for 5 days before operation (mean $\pm$ SE, 828 $\pm$ 137 ng/mg protein).

Fig. 2 shows a close relation between the calmodulin levels in Graves’ disease thyroids and the cell heights of the acinar epithelium. The mean and SE of values in a columnar cell predominant group were $1150 \pm 118$ ng/mg protein, which were significantly greater than those in a flat cell predominant (mean $\pm$ SE, $561 \pm 125$ ng/mg protein; $p<0.05$) or a cuboidal cell predominant group (mean $\pm$ SE, $596 \pm 40$ ng/mg protein; $p<0.001$). Serum TSH levels revealed no significant difference between any two groups.

![Fig. 1. Calmodulin levels in excised thyroid tissues. Tissues were obtained from 15 patients with nonfunctioning solitary nodule (normal tissues), 48 patients with Graves' disease treated with methimazole or propylthiouracil and 6 patients with Graves' disease administered T₃ (50 µg/day for 5 days) concurrently with methimazole. The brackets denote the mean $\pm$ SE. The shaded area indicates the normal $\pm$ 2SD of 15 normal values.](image-url)
There was no significant correlation between the tissue calmodulin levels and T₃, T₄, TSH, T₃/T₄ ratio determined at the time of operation, the weights of resected thyroid tissues or the degrees of exophthalmos.

Since the results were expressed as the amounts of calmodulin per mg of protein, the differences in calmodulin levels were certainly not related to those of total protein levels, but rather seemed to be specific for calmodulin. Similar results were also obtained when calmodulin contents were expressed as the calmodulin per mg wet weight. As the amounts of DNA recovered from 1 g wet weights of normal and Graves' disease thyroids were almost identical (normal: 7.1 ± 0.6 mg, n = 5; Graves': 6.0 ± 0.6 mg, n = 5; p > 0.05), similar results are also supposed to be obtained when the data are expressed on a per mg DNA basis.

Fig. 3 shows the localization of calmodulin, which was shown by means of immunofluorescence using anticalmodulin antibody.
modulin immunoreactivity in normal human thyroid tissue by immunofluorescence using anticalmodulin antibody. Calmodulin immunoreactivity was demonstrated in the apical border of thyroid epithelial cells in a form of granulated structure.

Discussion

The present study was performed to compare the calmodulin level in Graves' disease thyroid with that in normal thyroid. The calmodulin level was determined by specific radioimmunoassay. It was reported to be more accurate than phosphodiesterase assay because it allows measurement of calmodulin in the presence of EGTA and quantification of calmodulin on the basis of antigenic determinants rather than biological activity (Wallace et al., 1979; Sharma et al., 1978; Chafouleas et al., 1979). The results demonstrated that the total intracellular calmodulin level was significantly higher in Graves' disease thyroid than that in normal thyroid. Both the patients with Graves' disease and the patients from whom normal thyroids were obtained were euthyroid at the time of operation. However, the patients with Graves' disease had been treated with an antithyroid drug. We also measured the calmodulin levels in Graves' disease thyroids from patients administered T₃ at the daily dose of 50 μg for 5 days concurrently with an antithyroid drug, and these TSH levels were suppressed to 2.5±0.5 μu/ml (mean±SE) at the time of operation. In spite of the above treatment, the calmodulin level was also higher in Graves' disease thyroid, which was thought to be consistent with the T₃ nonsuppressibility known to exist in vivo.

Ca²⁺ has long been recognized as a key regulator of many physiological processes, such as muscle contraction, cell division and motility, neurotransmission, ion transport and secretion (Kretsinger 1979). It has become increasingly clear that the action of Ca²⁺ is mediated through a class of Ca²⁺-binding proteins. Among them, especially calmodulin which is most widely distributed and multifunctional appears to play a central role in the coordination of basic cellular activities (Kakiuchi et al., 1978; Wang and Waisman 1979; Means and Dedman 1980; Klee et al., 1980). Indeed, it was supposed that Ca²⁺/calmodulin was intimately involved in the regulation of cellular activities in the thyroid gland including cyclic nucleotide metabolism (Yagura et al., 1978). However, the potential significance of the increase in this protein in thyroid is not clear. A previous report has shown that the rise in the total intracellular calmodulin level in a transformed cell led to destablity of microtubules and resulted in a significantly reduced number and length of microtubules as compared with those observed in a non-transformed cell (Chafouleas et al., 1981). Colchicine and other drugs that promote microtubule disassembly enhanced the stimulation of DNA synthesis in a 3T3 cell by EGF (Friedkin et al., 1979; Otto et al., 1979) and the insulin- or serum-stimulated entry of chick embryo cell into the S phase of the cell cycle (Teng et al., 1977). Recently, it was reported that a rise in the intracellular level of calmodulin was coupled to the G1-S transition of the cell cycle, and that inhibition of the action of this protein by the anticalmodulin drug N-(4-aminobutyl)-5-chloro-2-naphthalenesulfonamide (W13) inhibited progression into S phase (Chafouleas et al., 1982). Thyroid growth stimulating antibodies were recently detected in sera of some patients with Graves' disease which induce the entry of thyroid epithelial cells into S phase (Drexhage et al., 1980), prior to which the phase intracellular calmodulin level was reported to be elevated (Chafouleas et al., 1982). It was also reported that the proportion of cells in S phase was larger in Graves' disease thyroid than that in normal human thyroid. It is also interesting that calmodulin levels in
Graves' disease thyroids in which the cell type of follicular epithelium is columnar cell predominant, which is supposed to be most stimulated by thyroid growth stimulating immunoglobulin, were significantly higher than those in a flat cell or a cuboidal cell predominant type. Taking the above facts into account, the rise in the total intracellular level of calmodulin might reflect a larger proportion of cells in S phase in thyroid tissue.

It was also reported that a change in the intracellular calmodulin level could influence on cAMP metabolism by regulating adenylate cyclase or phosphodiesterase activity. For example, the total calmodulin levels in liver cells from rats with streptozotocin-induced diabetes were significantly depressed and such changes might play a role in the alteration of cAMP metabolism known to exist in such pathological states (Smaoke and Solomon 1980). Since the intracellular cAMP level in Graves' disease thyroid was elevated (Kuzuya et al., 1980), the higher calmodulin level might also be related to such changes in cAMP metabolism.

Calmodulin was shown to be preferentially located in the apical border of thyroid epithelial cells, as well as actin (Miyagawa, 1982) and myoglobin (Miyoshi, 1983). The enrichment of calmodulin in the apical border might suggest the involvement of calmodulin in a process of exo- or endocytosis.

**References**


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