Characteristics of Antibodies to Calmodulin in Patients with Graves' Disease

YOSHIYUKI NAKAJIMA, YOSHIHIRO KAJITA, MASAO ISHIDA, YASUAKI URA, YUKIO OCHI*, TAKASHI HACHIYA** AND HIROYOSHI HIDAKA***

Department of Internal Medicine, Nantan General Hospital, Yagi, Kyoto 629-01, Japan
* Central Clinical Laboratory, Shiga University of Medical Science, Otsu, Shiga 520-21, Japan
** Internal Medicine, Kyoto Prefectural University of Medicine, Kyoto 602, Japan
*** Department of Pharmacology, Mie University Medical School, Tsu, Mie 514, Japan

Abstract

We detected an antibody to calmodulin (CaM) in sera from patients with Graves' disease. Four sera out of 300 from patients with Graves' disease demonstrated increased CaM binding activity as compared with 300 sera from normal subjects, while no binding activity was detected in sera from autoimmune thyroiditis. The binding could be demonstrated as due to the antibody to CaM by the double antibody method, polyethyleneglycol method, gel filtration and radioimmuonelectrophoresis, respectively. These antibodies were thought to be polyclonal immunoglobulins (IgG and/or IgA).

CaM has proven to be a poor antigen because of the structural identity of CaM from different species. The incidence of the antibody to CaM in Graves' disease is low and the pathophysiological significance of this antibody to CaM had remains obscure.

The pathogenesis of Graves' disease is thought to be a thyroid stimulator in the patient's serum. Historically, LATS by Adams (1956) is well known but it is not detected in all patients with Graves' disease (McKenzie 1968; Chopra et al., 1970), thereafter, several methods for measuring thyroid stimulators have been developed (Shishiba et al., 1973; Onaya et al., 1973; Orgiazzi et al., 1976). These stimulators have been proved to be the antibodies that bind to thyroidal antigens.

Recently, a TSH receptor assay system was developed (Manley et al., 1974; Smith and Hall 1974). Using these systems it has been reported that Graves' IgG inhibited the binding of labelled TSH to its receptor. Consequently, Graves' IgG is thought to be an antibody to TSH receptor, and this IgG is thought to stimulate the thyroid gland
in the same manner as TSH. However, TSH-binding inhibitor IgG in some sera from Hashimoto’s disease (Endo et al., 1978; Konishi et al., 1983) and neonatal transient hypothyroidism due to maternal TSH binding inhibitor IgG (Matsuura et al., 1980; Takasu et al., 1984) have also been reported. On the other hand, because antibodies to thyroglobulin (Roitt et al., 1956) and to thyroid microsomal antigen (Roitt et al., 1964) in Graves’ disease were reported, Graves’ disease is thought to be a kind of an autoimmune disease, similar to Hashimoto’s disease.

Calmodulin (CaM) is known as a general regulator of cell function and CaM is ubiquitous in animal cells (Kitajima et al., 1983). Recently we found CaM involved in the TSH receptor of thyroid (Nakajima et al., 1987). We also found the antibody to CaM in sera from patients with Graves’ disease. We here describe the results of these experiments.

Materials and Methods

1) Materials

Highly purified CaM from bovine brain was prepared as previously described (Hidaka et al., 1979; 1980). 125I-CaM preparation was purchased from Amersham (U.K.). This CaM is purified from rat testis and the specific activity of 125I-CaM is 75 μCi/μg.

2) Patients

A LATS (long acting thyroid stimulator) assay was performed by a minor modification of the method of McKenzie (Ochi et al., 1972). A TRAb (TSH receptor antibody) assay was performed with a commercially available TSH receptor assay kit supplied by Japan Travenol Co. as described previously (Kajita et al., 1984). Less than 15% was postulated as the normal range.

30 sera from Graves’ disease with LATS activity, 270 sera from Graves’ disease without LATS activity, 300 sera from Hashimoto’s disease, 10 sera from subacute thyroiditis, 8 sera from silent thyroiditis, 25 sera from thyroid cancer, 16 sera from systemic lupus erythematosus (SLE), 6 sera from progressive systemic sclerosis (PSS) and 8 sera from myeloma were examined. The diagnosis was made using standard clinical and laboratory criteria. As a control, 300 sera from normal adults were examined.

3) Methods for detection of antibody to 125I-CaM

In the routine assay, 0.85 ml of assay buffer (0.05 M phosphate buffer, pH 7.8), 0.1 ml of 125I-CaM (about 20,000 cpm) and 0.05 ml of test serum were mixed. After incubation for 72 hours at 4°C, 1 ml of cold polyethylene glycol (PEG, No. 6,000) solution (25% w/v in 1 M NaCl) was added to the assay mixture. After mixing well, the tubes were centrifuged at 1,500 × g for 30 min at 4°C and counted after the removal of the supernatant.

In the double antibody experiment, goat antiserum to human gamma-globulin (0.05 ml) (made by our laboratory) was added as the second antibody. After the first incubation for 72 hours in a medium containing 0.1 ml of 125I-CaM, 0.01 ml of test serum and assay buffer in a total volume of 0.95 ml, 0.05 ml of the second antibody was added and incubated overnight at 4°C. Thereafter, the assay tube was centrifuged and counted.

In a Protein A adsorbent experiment, 0.005 ml of test serum and 0.1 ml of 125I-CaM and 0.9 ml of assay buffer were mixed. After incubation for 72 hours at 4°C, 0.1 ml of Pansorbin (Calbiochem) was added to the assay mixture. After a 2 hour incubation at room temperature, the immune complexes were pelleted by centrifugation at 1,500 × g for 15 min at 4°C. The pellets were washed in assay buffer, centrifuged as above and counted.

4) Precipitation of 125I-CaM by patient’s serum

Precipitation of 125I-CaM by patient’s serum diluted by normal human serum (NHS) was examined. 0.1 ml of the mixture of both patient’s serum and normal human serum was incubated for 72 hours at 4°C in a total volume of 1.0 ml by adding the assay buffer, followed by the addition of 1.0 ml of cold PEG solution (25% w/v in 1 M NaCl). The resulting precipitate was collected by centrifugation at 1,500 × g for 30 min and counted.

5) Effect of unlabelled CaM on the binding of 125I-CaM to patient’s serum

In the routine assay for detection of antibody to 125I-CaM, unlabelled CaM was added over the range of 10^-6-10 μg/tube.
6) Determination of immunoglobulin class and light chain type of the antibody by radioimmunoprecipitation

This experiment was performed by the double antibody method mentioned above. Instead of goat antiserum to human gamma-globulin, rabbit antiserum to human IgG, IgM or IgA (MBL, Tokyo) (0.01 ml) was incubated for 72 hours at 4°C.

In order to determine the light chain type of the antibody for CaM, rabbit antiserum to human kappa or lambda chain (MBL, Tokyo) (0.1 ml) was added to the previously incubated medium of both 125I-CaM and patient's serum (0.005 ml). After incubation for 24 hours at 4°C, 0.1 ml or sheep antiserum to rabbit gamma-globulin (MBL) was added to obtain the precipitate.

7) Analysis of binding protein by gel-filtration

After incubation of 0.5 ml of patient's serum (K.K.) and 125I-CaM (about 200,000 cpm) with or without unlabelled CaM (100 µg/tube) for 72 hours at 4°C, gel-filtration was performed with a Sephacryl S-300 (Pharmacia Fine Chemicals) column (2.5×44 cm) that was equilibrated with 10 mM tris-HCl buffer, pH 7.5 containing 50 mM NaCl, 3 mM NaN3.

8) Radioimmunoelectrophoresis (RIE)

RIE was performed to determine the immunoglobulin class of CaM binding gamma-globulin (Yagi et al., 1962; Kajita et al., 1978). 125I-CaM and patient's serum (0.1 ml) were preincubated for 72 hours at 4°C before RIE. The precipitated line was developed by rabbit antiserum against human IgG, IgM, IgA, kappa chain and lambda chain, respectively and radioautography was performed.

Results

1) Detection of antibody to 125I-CaM

The PEG precipitation of radioactive CaM
of 4.4 to 7.0 percent (mean±2SD) was observed in sera from 300 normal control.

One out of 30 LATS positive sera from patients with Graves' disease and 3 out of 270 sera without LATS activity showed increased binding activity compared to that of 300 sera from control subjects. None of the sera from Hashimoto's disease (300), subacute thyroiditis (10), silent thyroiditis (8) or thyroid cancer (25), as far as we examined, showed any increased binding activity (Fig. 1). No sera from systemic lupus erythematosus, progressive systemic sclerosis or myeloma showed any binding activity (data not shown). The same results were obtained by using double antibody and Protein A adsorbent (data not shown).

2) Precipitation of 125I-CaM by patient's serum

The binding of 125I-CaM with patient's serum diluted by NHS was examined by the PEG method. The radioactivity precipitated increased proportionally to the amount of serum (Fig. 2).

3) Effect of unlabelled CaM on the binding of 125I-CaM to patient's serum

The binding of 125I-CaM to patient's serum was inhibited dose-dependently by adding unlabelled CaM over the range of 10^-6-10 μg/tube (Fig. 3).

4) Determination of immunoglobulin class and light chain type of the antibody

Using the double antibody method, 3 patients' sera (K.K., T.U. and K.F.) were precipitated by the antisemur to IgG and IgA; one patient (M.S.) only by the antisemur to IgG. All 4 patients sera were precipitated significantly by the antibody to kappa type, but only slightly by the anti-

Fig. 2. Effect of amount of patient serum on the binding of 125I-CaM

Reaction volume: 1.0 ml, incubation conditions: 72 hours at 4°C. All values shown are the mean for three experiments.

Fig. 3. Effect of unlabelled CaM on the binding of 125I-CaM to patient serum

Reaction volume: 1.0 ml, incubation conditions: 72 hours at 4°C. All values shown are the mean for three experiments.
Table 1. Specific binding of $^{125}$I-CaM with immunoglobulin of patients sera (immunoglobulin class & light chain type). Precipitated radioactivity was examined by the double antibody method. All values shown are the mean for three experiments.

<table>
<thead>
<tr>
<th>Rabbit serum</th>
<th>B/T (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal serum</td>
<td>K.K.</td>
</tr>
<tr>
<td>Anti-IgG</td>
<td>3.1</td>
</tr>
<tr>
<td>Anti-IgA</td>
<td>4.0</td>
</tr>
<tr>
<td>Anti-IgM</td>
<td>3.3</td>
</tr>
<tr>
<td>Anti-$K^*$</td>
<td>4.3</td>
</tr>
<tr>
<td>Anti-$\lambda^*$</td>
<td>4.2</td>
</tr>
</tbody>
</table>

*Sheep antiserum to rabbit gamma-globulin was added for identification of light chain type of CaM antibody. K.K.: LATS positive patient (520% response at 24 hours).

body to lambda type (Table 1).

5) **Experiment using gel-filtration**

The elution pattern of $^{125}$I-CaM with patient's serum (case K.K.) on a Sephacryl S-300 column is shown in Fig. 4. CaM has a molecular weight of about 16,700. Thus, the eluted radioactivity was found mainly behind the third peak (albumin fraction) in the NHS pre-incubated with $^{125}$I-CaM.

In contrast the peak of eluted radioactivity was found behind the void volume and before the second peak in the patient's serum. When $^{125}$I-CaM and patient's serum were preincubated with unlabelled CaM (100 $\mu$g/tube), these radioactivities disappeared completely.

---

**Fig. 4.** Gel-filtration of $^{125}$I-CaM containing patient's serum using Sephacryl S-300

$^{125}$I-CaM with patient's serum (K.K.) was applied to the column (4°C, 12 ml/h) and 3 ml fractions were collected.

NHS: normal human serum
6) Radioimmunoelectrophoresis

As shown Fig. 5, autoradiography clearly identified the binding globulin of patient (K. K.) to be IgG, IgA. The radioactivity in the antibody for kappa chain was significant, but that in the antibody for lambda chain was faint.

7) TRAb activity in Graves’ patients with positive CaM antibody

TRAb activity in 4 sera was 60.2% (K. K.), 26.2% (T. U.), 41.6% (M. S.) and 75.0% (K. F.), respectively. One LATS positive case (K. K.) showed high TRAb activity. Three LATS negative cases also showed positive TRAb activity.

Discussion

CaM is a single polypeptide consisting of 148 amino acids with a molecular weight of 17,000 and acidic protein distributed in almost all tissues in the animal and plant kingdom (Kitajima et al., 1983). It is generally believed that CaM is virtually identical with the sequence in animal cells from the sequence homology of CaM from bovine brain (Watterson et al., 1980), bovine uterus (Grand and Perry, 1978), and rat testis (Dedam et al., 1978). Because of the lack of species-specificity of this protein with respect to antigenicity, the difficulty in producing an antibody to CaM has been reported (Wallance and Cheung, 1979, Van Edlik and Watterson, 1981, Kitajima et al., 1983).

Recently, we found the involvement of CaM in the TSH receptor of thyroid (Nakajima et al., 1987), and then we found the antibody to CaM in sera from patients with Graves’ disease. Four sera out of 300 from patients with Graves’ disease had increased CaM binding immunoglobulins but there were no CaM binding immunoglobulins in sera from autoimmune thyroiditis. These sera bound to the labelled CaM dose-dependently and this binding was inhibited by the addition of unlabelled CaM. CaM binding immunoglobulins were confirmed to be the polyclonal IgG and/or IgA (1 was a IgG type and 3 were IgG and IgA types). Radioimmunoelectrophoresis also revealed the binding immunoglobulins for CaM. Faint radioactivity by lambda antibody in both radioimmunoprecipitation and autoradiogra-
phy may be due less to the antibody titer of lambda antibody than to the kappa antibody. When the labelled-CaM was mixed with the patient’s serum, an immune complex formation was demonstrated by gel-filtration on Sephacryl S-300. The radioactivity before the second peak (gamma-globulin fraction) is thought to be an immune complex between the labelled-CaM and IgA or IgG. The radioactivity behind the void volume is thought to be an aggregation of these immune complexes.

Rousset et al. (1983) have reported immunoglobulins which bind to tubulin in sera from patients with autoimmune thyroid disorders, especially Graves’ disease and Hashimoto’s thyroiditis. Kajita et al. (1983) and Akamizu et al. (1984) have also reported auto-antibodies to bovine TSH in patients with Graves’ disease, and Eto et al. (1984) have reported them in patients with Hashimoto’s thyroiditis.

The TSH receptor activity in 4 Graves’ sera with positive CaM antibody was examined. One LATS positive case showed positive TRAb activity as in the previous report (Kajita et al., 1984). TRAb activity was positive in all 3 LATS negative cases. Neither LATS activity nor TRAb activity may have any direct relation with a CaM antibody. Now, with the detection of antibodies in Graves’ disease in the present investigation, several antibodies seem to be present in autoimmune thyroid disease, making the pathogenesis of this disease more diversified and complicated. The presence of an antibody to CaM in Graves’ disease may play a role in the pathogenesis of Graves’ disease, because CaM has proven to be a poor antigen.

Acknowledgements

The authors wish to thank Mrs Tomoko Ishizumi and Miss Kyoko Murai and Dr. M. G. Karmarkar (All India Institute of Medical Science) for their help in the preparation of this manuscript.

This work was partly supported by Grand-in Aid No. 59570478 for Scientific Research (C) in Japan.

References


