Vol. 144, No. 1, 1987 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1987 Pages 191-197

ANTIBODY THAT RECOGNIZES CONFORMATIONS OF CALMODULIN IN THE SERUM FROM PATIENT WITH CHRONIC ACTIVE HEPATITIS

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Received February 16, 1987

SUMMARY. According to the effects of Ca^{++} on the reactivity, anti-calmodulin antibody in the sera of patients with autoimmune chronic active hepatitis was classified into three types, which were tentatively designated as type 1, 2 and 3. Type 1 antibody reacted with calmodulin only in the presence of free Ca^{++}. Binding of type 2 antibody to calmodulin was inhibited by the presence of free Ca^{++}. Type 3 antibody reacted with calmodulin regardless of the presence or absence of free Ca^{++}. Thus the sera contained the populations of the antibody which recognized the different conformations of calmodulin molecule.

Calmodulin (CaM) is an intracellular Ca^{++}-binding protein and involved in the regulation of the activities of Ca^{++}-dependent enzymes. The wide distribution and the high degree of sequence homology among species made it difficult to produce the antibody to CaM (CaM antibody). Nevertheless, several laboratories have succeeded in producing the anti-sera by immunization with native or modified CaM (1-6). Recently we reported the occurrence of CaM antibody in the sera of patients with liver diseases and systemic lupus erythematosus (7).

The conformational change of CaM molecule induced on binding of Ca^{++} was suggested on the basis of NMR (8), circular dichroism

Abbreviations used are : CaM, calmodulin ; HRP, horseradish peroxidase ; TBS, Tris-buffered saline ; Ca-TBS, TBS containing 1mM $CaCl_2$; EGTA-TBS, TBS containing 1mM EGTA ; ELISA, enzyme-linked immunosorbent assay ; BSA, bovine serum albumin.

0006-291X/87 $1.50

(9), fluorescence (10,11), chemical modification (12), X-ray analysis (13), and limited proteolysis studies (14). Harper reported the production of the antibody which recognized only Ca^{++}-bound CaM (5). The antibody raised against Tetrahymena CaM by McCartney et al. exhibited higher avidity for CaM in the presence of Ca^{++} than in the presence of EGTA (6). In the present report, we showed that the sera positive for CaM antibody contained the populations of the antibody which recognized the conformations of CaM molecule.

MATERIALS AND METHODS

Reagents. Bovine brain CaM, purified by the method of Teo et al. (15), was purchased from Amano Co.(Tokyo). Horseradish peroxidase (HRP)-labelled rabbit anti-human IgG was the product of Cappel Lab.(Malvern, PA). Agarose coupled with CaM from bovine brain (CaM-agarose) was purchased from Sigma Chemical Co.(St Louis, MO).

Serum. The sera studied in the present study were collected from 13 healthy subjects and 4 patients with autoimmune chronic active hepatitis.

CaM-agarose column chromatography. CaM-agarose was packed in the column (1.5 x 6cm), and then equilibrated with 0.135M NaCl/0.015M Tris-HCl (pH 7.6) (TBS) containing either 1mM $CaCl_2$ (Ca-TBS) or EGTA (EGTA-TBS). Five μl of 100mM $CaCl_2$ or EGTA was added to 500μl of serum positive for CaM antibody. The mixture was then applied to the column. The column was washed with the equilibration buffer until no antibody was detected in the eluate, and then eluted with EGTA-TBS or Ca-TBS, which was followed by 0.1M glycine/HCl (pH 2.5) at the flow rate of 2.0ml/min. The eluate was collected in 3.0ml fractions, and tested for CaM antibody as described below. The fractions eluted with glycine/HCl were neutralized to pH 7.6 with NaOH before the assay of the antibody.

Enzyme-linked immunosorbent assay (ELISA) of CaM antibody. ELISA was performed as described previously (7). Briefly, the wells of microtiterplate (Nunc Immunoplate I-96F) were coated with bovine brain CaM, and washed with TBS containing 0.05%(V/V) Tween 20 (washing buffer). The free binding sites were blocked by incubating the wells with 1%(W/V) bovine serum albumin (BSA)/ 0.15M Tris-HCl (pH 7.6) (blocking buffer) overnight at 4°C. The antibody in the serum samples or the fractions from CaM-agarose column was assayed under the following two conditions. 1) Assay in the presence of Ca^{++} : Ninety μl of each fraction or 1/250 diluted serum sample in TBS was mixed with 10μl of the blocking buffer containing 20mM $CaCl_2$, put into the well and incubated overnight at 4°C. The wells were washed six times with the washing buffer containig 1mM $CaCl_2$. IgG bound to CaM fixed to the wells was determined with HRP-labelled rabbit anti-human IgG, and expressed in the absorbance at 492nm as described previously (7). 2) Assay in the absence of Ca^{++} : Ten μl of 20mM EGTA was added to 90μl of each fraction eluted or 1/250 diluted serum sample in TBS. The subsequent assay procedures were the same as described above except that both the blocking buffer and the washing buffer

contained 1mM EGTA instead of 1mM $CaCl_2$.
Effect of Ca^{++} on binding of CaM antibody to CaM. In CaM-agarose column chromatography of the sera, the fractions containing the peak antibody activity were collected. Various amounts of $CaCl_2$ and/or EGTA in 10μl were then added to 90μl samples of each fraction to give various concentrations of free Ca^{++}. The concentrations of free Ca^{++} were calculated according to Bartfai (16). BSA was also added to the samples to give a final concentration of 1%. The mixtures were put into the wells coated with CaM, and incubated overnight at 4°C. Each well was washed with the washing buffer in which the concentration of free Ca^{++} was maintained by 1mM $CaCl_2$-EGTA buffer system in the same concentration as that in the incubation medium for binding of the antibody. The antibody bound to CaM was determined with HRP-labelled rabbit anti-human IgG as described above.

RESULTS

Fig.1 shows the titres of CaM antibody in the control subjects and four patients. Addition of $CaCl_2$ to the asssay mixtures increased the titres. In each of two patinets, SO and MS, a marked difference was observed between the titres obtained by ELISA in the presence of $CaCl_2$ and EGTA. These findings suggested that the sera contained the antibodies which differentiated between Ca^{++}-bound and Ca^{++}-free forms of CaM molecule, and prompted us to isolate them by CaM-agarose column chromatography.

The sera from two patients, AO and HY, were applied to CaM-agarose columns equilibrated with Ca-TBS and EGTA-TBS, respectively. The columns were eluted with EGTA-TBS or Ca-TBS, and then glycine/HCl (pH 2.5). The eluate was tested for the antibody by ELISA in the presence of excess free Ca^{++} or EGTA. In EGTA-TBS eluate, the antibody was detected in the presence of excess free Ca^{++}, but not in the presence of excess EGTA (Fig.2). In Ca-TBS eluate, the antibody was detected in the presence of excess EGTA, but not in the presence of excess free Ca^{++} (Fig.3). In glycine/HCl eluate, the antibody was detected regardless of the presence or absence of free Ca^{++} (Fig.2 and 3). Fig.4 shows the effects of free Ca^{++} concentrations on the binding to CaM of the antibody in the EGTA-TBS, Ca-TBS and glycine/HCl eluate. The

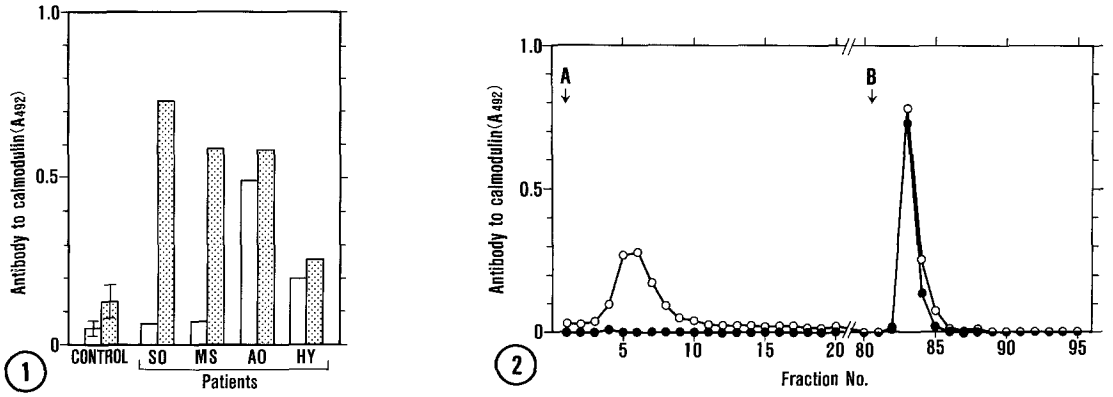

Fig.1. Anti-calmodulin titres in the control subjects and the patients.

Open and shaded columns represent the titires obtained by ELISA in the presence of 1mM EGTA and $CaCl_2$, respectively, as described in MATERIALS AND METHODS. In the control subjects (CONTROL), the columns represent the means ± SD of the titres in 13 healthy subjects.

Fig.2 . Elution of the anti-calmodulin antibody from calmodulin-coupled agarose column equilibrated with Tris-buffered saline (TBS) containing 1mM $CaCl_2$.

The serum from a patient, AO, was applied to the column. The column was eluted with TBS containing 1mM EGTA (arrow A) and then 0.1M glycine/HCl buffer (pH 2.5) (arrow B). The eluate was tested for the antibody in the presence of free Ca^{++} (○) or excess EGTA (●) as described in MATERIALS AND METHODS.

binding of the antibody in the EGTA-eluate (type 1 antibody) was completely dependent on free Ca^{++}. In contrast, the binding of the antibody in Ca-TBS eluate (type 2 antibody) was inhibited by

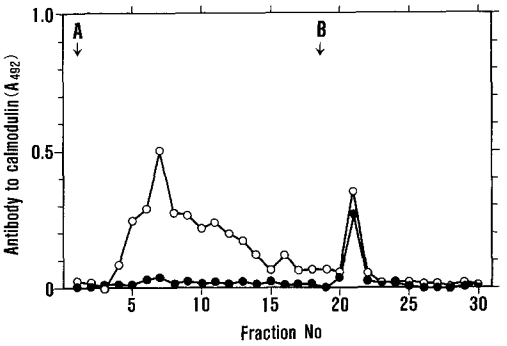

Fig.3 . Elution of anti-calmodulin antibody from calmodulin-coupled agarose column equilibrated with Tris-bufferd saline (TBS) containing 1mM EGTA.

The serum from a patient, HY, was applied to the column. The column was eluted with TBS containing 1mM $CaCl_2$ (arrow A) and then 0.1M glycine/HCl buffer (pH 2.5) (arrow B). The eluate was tested for the antibody in the presence of excess EGTA (○) or free Ca^{++} (●) as described in MATERIALS AND METHODS.

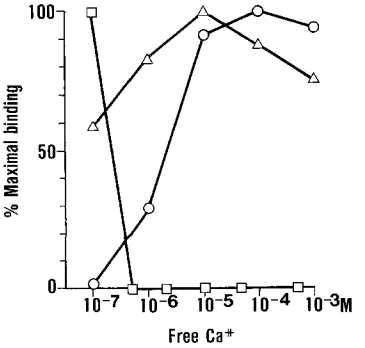

Fig 4. Effects of free Ca^{++} concentrations on the binding to calmodulin of the antibodies eluted from calmodulin-coupled agarose columns.

The antibodies were isolated by calmodulin-coupled agarose column chromatography as shown in Fig. 2 and 3. Effects of free Ca^{++} concentrations on their binding to calmodulin were studied as described in MATERIALS AND METHODS. The antibody binding was expressed in % of the maximal binding.

○: The antibody eluted with Tris-buffered saline containing 1mM EGTA in the experiment shown in Fig.2.

□: The antibody eluted with Tris-buffered saline containing 1mM $CaCl_2$ in the experiment shown in Fig.3.

△: The antibody eluted with 0.1M glycine/HCl buffer (pH 2.5) in the experiment shown in Fig.2.

free Ca^{++}. The antibody in glycine/HCl eluate (type 3 antibody) bound to CaM regardless of the presence or absence of free Ca^{++}. With the increase in the concentration of free Ca^{++}, however, type 3 antibody binding to CaM increased.

DISCUSSION

There is cumulative evidence for Ca^{++}-induced conformational change of CaM molecule. On limited proteolysis of CaM by trypsin, the cleavage occurred at Arg 106 and Arg 37 under Ca^{++}-free condition (14). In the presence of Ca^{++}, trypsin attacked Lys 77, not the Arg residues (14). These findings indicated that, in Ca^{++}-bound CaM, the domains containing the Arg residues were buried and that containing the Lys residue was exposed to the solvent. The domain which was exposed regardless of the presence or absence of Ca^{++} was also suggested. Fluorescence and chemical modification studies suggested that Tyr 99 was exposed to the solvent, whether Ca^{++} was present or not (11,12). CaM antibodies

demonstrated in the present study were presumably directed against the different domains which were suggested by these studies.

In the present study, we tentatively classified the antibodies into three types. Type 1 antibody bound to CaM only in the presence of free Ca^{++}, and presumably reacted with the domain which was exposed on binding of Ca^{++}. This was supported by the finding that the antibody binding to CaM increased in the range of free Ca^{++} concentration (10^{-6} to 10^{-4}M), where fluorescence change and circular dichroism change of CaM molecule occurred (9-11). The binding of type 2 antibody was inhibited by the presence of free Ca^{++}. This antibody presumably bound to the domain which was buried in the presence of Ca^{++}. Type 3 antibody bound to CaM regardless of the presence or absence of Ca^{++}. This antibody bound to the domain which was exposed regardless of the presence or absence of Ca^{++}.

Immunochemical methods have been useful for elucidation of the function of CaM in the cell. CaM antibodies that recognize conformations of CaM are easily prepared from the patients' sera and will be useful for these purposes.

ACKNOWLEDGMENTS. This work was partly supported by the grants from Japanese Ministry of Education and Yamanouchi Foundation for Disease and Metabolism.

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