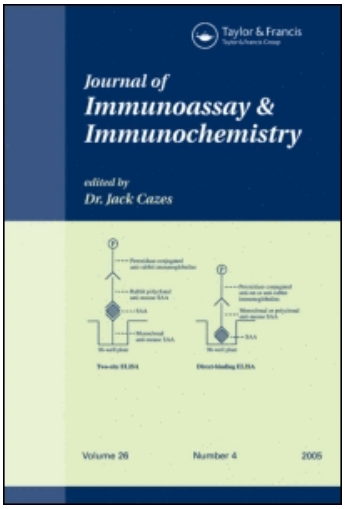

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Production of a High Affinity Antibody Specific to the Calcium-Free-Form of Calmodulin, Using N-Acetyl-muramyl-L-alanyl-D-isoglutamine-calmodulin Conjugate

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PRODUCTION OF A HIGH AFFINITY ANTIBODY SPECIFIC TO THE CALCIUM-FREE-FORM OF CALMODULIN, USING N-ACETYL-MURAMYL-L-ALANYL-D-ISOGLUTAMINE-CALMODULIN CONJUGATE

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ABSTRACT

Covalent coupling of N-acetyl-muramyl-L-alanyl-D--isoglutamine (MDP) to calmodulin was used in order to enhance its immunogenicity. Rabbit antibodies against calmodulin were obtained, interacting with the calcium--free-form of calmodulin. The radioimmunoassay was developed using the whole sera (titre 1:20,000) having a sensitivity of 20 pg per tube (50% displacement 500 pg/assay tube). The radioimmunoassay shows the same concentrations of calmodulin in rat brain homogenate (4.9 ± 0.86 µg/mg protein), bovine neurosecretosomes (0.77 ± 0.10 µg/mg protein), and bovine neurohypophysial secretory vesicles (0.05 ± 0.01 µg/mg protein) as the phosphodiesterase activation method.

(KEY WORDS: calmodulin, MDP, radioimmunoassay)

INTRODUCTION

Calmodulin - by far the most widely distributed calciprotein is considered to be a poor antigen, maybe because

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of its structural and functional conservativeness throughout molecular evolution. There are in the literature several reports (1-7) describing preparation of antibodies against calmodulin and development of radio- or enzyme-linked immunoassays. However, there is a need for antisera with higher titres and greater affinity.

In this report we describe a preparation of antibodies to calmodulin using a new type of antigen for immunization, i.e. a conjugate of N-acetyl-muramyl-L-alanyl-D-isoglutamine and calmodulin, and the development of a sensitive radioimmunoassay, as well as data about calmodulin content in secretory vesicles and neurosecretosomes from ox neurohypophyses.

MATERIALS AND METHODS

Calmodulin was purified according to ref. (8) from bovine brain. The purification was checked by the phosphodiesterase activation method (9). The final product used for immunization showed about 5% contamination by a low molecular weight component on SDS-electrophoresis. For the booster injections calmodulin was purified using HPLC. As a standard for radioimmunoassay we used calmodulin purchased from Fluka. ^{125}I-calmodulin was either the preparation made by us, iodinated using the Iodogen method (10), or a commercial product from New England Nuclear. Troponin and Thyroglobulin were from Sigma. N-acetyl-muramyl-alanyl-D--isoglutamine (MDP) was a generous gift from M.Flegel, Léčiva, Prague.

Preparation of three types of antigen

i) Peroxidized calmodulin was prepared according to (7) starting from 20 mg of calmodulin. After freeze-drying the peroxidized compound was dissolved in 2 ml of physiological saline and stored at -20°C.

ii) Thyroglobulin-calmodulin conjugate was prepared using carbodiimide (11), with 11.5 mg of thyroglobulin and

2.7 mg of calmodulin coupled. After dialysis the solution was stored at -20°C.

iii) MDP-calmodulin conjugate was prepared also using carbodiimide. 2 mg of MDP in 0.1 ml of water reacted with 2 mg of carbodiimide for 30 min. Then 8.9 mg of calmodulin in 0.5 ml of aqueous solution, pH 8.5, was added. This mixture was stirred at room temperature for 24 h, then dialyzed against water, pH 8 (adjusted using $NaHCO_3$). After dialysis the conjugate was stored frozen at -20°C. The conjugate of HPLC-purified calmodulin with MDP was prepared similarly using the same ratios of reagents.

Immunization

Altogether 8 rabbits were immunized in the following way: I. 3 rabbits (A, B, C) were immunized using peroxidized calmodulin (antigen (i)) on days 1, 7, 9, 13, 15, 17, 35, 49, and MDP-calmodulin HPLC conjugate (antigen (iii)) on days 139 and 155. II. 2 rabbits (D, E) were immunized using thyroglobulin-calmodulin conjugate (antigen (ii)) on days 1, 7, 13, 17, and MDP-calmodulin-HPLC conjugate on days 139 and 155. III. 3 rabbits (F, G, H) were immunized using MDP-calmodulin conjugate (antigen (iii) on days 1, 7, 9, 13, 17, 35, 49, and using MDP-calmodulin-HPLC on days 139 and 155. All antigens used were diluted in such a way that the animals got about 250 µg of calmodulin per immunization. The sera were harvested on days 28, 44, 58, 150, and 167.

Radioimmunoassay

The antibodies were detected using iodinated calmodulin either prepared commercially or prepared by us using the Iodogen method. Conditions for testing the presence of antibodies as well as for the radioimmunoassay were as follows: 0.3 ml of appropriately diluted antiserum, 0.1 ml of ^{125}I-calmodulin (4,000-10,000 cpm) and 0.2 ml of standard calmodulin or buffer or unknown sample were incubated for 48 h at 4°C. Separation of bound and free calmodulin was done using the double antibody technique. After 16-24 h in-

cubation with the second antibody the tubes were centrifuged at 3,000 rpm for 25 min at 4°C. Supernatant was aspirated and the sediment was counted for radioactivity. For diluting the antibody and ^{125}I-calmodulin the following buffer was used: 0.1 M Tris-HCl, 1 mM ethylene-glycol-bis(α-aminoethyl ether)N,N^{1}-tetraacetic acid (EGTA), 0.2% bovine serumalbumin (BSA), pH 7.8. The standard calmodulin and samples were diluted in 20 mM N-Tris(Hydroxymethyl)methyl-2-aminoethane Sulfonic Acid (TES), 0.2% BSA, pH 7.0. In some experiments $CaCl_2$ was added to a final free concentration of 0.1 mM.

Sample preparation

The rat brain homogenate and extract were prepared as follows: rat brain was homogenized in 10 volumes of 190 mM sucrose, 25 mM EGTA and 20 mM TES, pH 7, by a teflon-to-glass homogenizer at 2,000 rpm, 6 strokes up and 6 strokes down. Part of the homogenate was stored frozen, part was centrifuged for 30 min at 100,000 g_{av}. The supernatant was divided into two parts: the first was stored frozen as such, the second was quickly heated to 97°C, cooled, and centrifuged for 5 min. in a Beckman microfuge, and the supernatant was stored at -20°C. Neurosecretory vesicles were prepared by the method of Gratzl et al. (12). Neurohypophysial nerve endings were prepared by trypsinization followed by centrifugation at 700 G for 10 min. and further centrifugation of the supernatant at 1,700 G for 25 min. (13).

RESULTS

The results of immunization are given in Table 1. Rabbits immunized initially using peroxidized calmodulin (A, B, C) did not respond by antibody formation, nor after a booster of MDP-calmodulin conjugate. All other rabbits immunized using MDP-calmodulin conjugate produced antibodies to calmodulin. The titres of the sera varied from 1:40 to

TABLE 1

DILUTIONS OF THE SERA GIVING 50% BINDING OF ^{125}I-CALMODULIN (a- commercial preparation of ^{125}I-calmodulin, b- Iodogen iodinated calmodulin; 0 means:no binding was detected even at the 1:5 dilution of the antisera, n.d. denotes not determined).

rabbit	harvest day				
	I.	II.	III.	IV.	V.
	28.	44.	58.	150.	167.
A,B,C	0[a,b]	0[b]	0[a,b]	0[a]	0[a]
D	0[b]	n.d.	n.d.	1:10[a]	1:50[a]
E	1:20[b]	n.d.	n.d.	1:20000[a]	1:1500[a]
F	1:40[b]	1:20[b]	n.d.	1:1000[a]	1:160[a]
G	1:200[b]	1:200[b]	1:300[a]	1:300[a]	1:50[a]
H	1:500[b] 1:2000[a]	1:300[b] 1:2000[a]	1:700[a]	1:1500[a]	1:300[a]

1:20,000. However, the highest titre was obtained after a booster injection and changing the type of antigen in rabbit E. All the antisera (i.e. antisera from all 5 rabbits (D-H) from different bleedings) obtained had high affinity to the calcium-free form of calmodulin as demonstrated in Fig. 1 on the examples of antisera from rabbits E and H. In the presence of 0.1 mM calcium there was a low degree of binding of radioactive calmodulin to antibodies and this binding was difficult to displace using cold calmodulin. Cross-reactivity with troponin was very low - 4,500-5,000

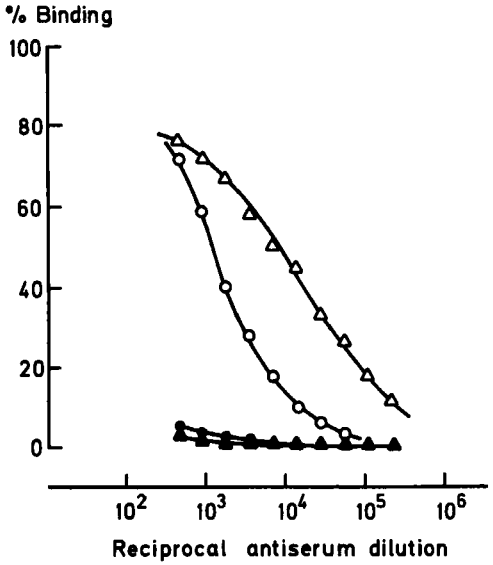

FIGURE 1

Effect of Ca^{2+} on ^{125}I-calmodulin binding to antibodies in the sera of rabbits E and H from the 4th and 2nd harvest resp.

Δ , O binding to E-IV and H-II serum (see table I), resp., in the absence of calcium (1 mM EGTA),

▲ , ● binding to E-IV and H-II serum, resp., in the presence of 10^{-4}M Ca^{2+}.

times more troponin (by weight) was necessary to obtain the same degree of displacement, but the displacement curve was parallel to that of calmodulin (Fig. 2). The displacement curves using rat brain extract boiled and unboiled and rat brain homogenate were parallel to that of standard calmodulin as well (Fig. 2). The sensitivity region of a radioimmunoassay using antisera from different bleedings of rabbits E, F, G, and H was 0.2-100 ng per tube (50% displacement of ^{125}I-calmodulin by non-radioactive calmodulin was 18-60 ng per tube) in average. The sensitivity region of a radioimmunoassay using serum E-IV, having the titre 1:20,000, was 5-10 times lower, i.e. 20-10,000 pg per tube (50% displacement 500 pg per tube).

The reliability of the method was tested by measuring the quantities of calmodulin in rat brain homogenate, bovine neurosecretosomes and bovine neurosecretory vesicles.

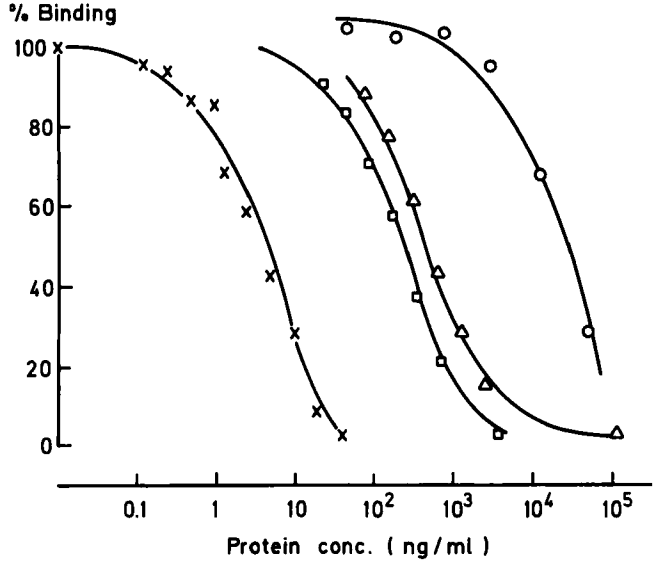

FIGURE 2

Immunological cross-reactivity of calmodulin (x-x) troponin (o-o), rat brain homogenate (Δ-Δ) and extract (□-□).

TABLE 2

CALMODULIN CONTENT IN SAMPLES measured using radioimmunoassay and phosphodiesterase activation method (μg/mg protein)

	RIA	PDE
rat brain homogenate	4.90 ± 0.86 (n=4)	5.96 (n=2)
secretory vesicles*)	0.05 ± 0.01 (n=4)	0.09 ± 0.01 (n=6)
neurosecretosomes*)	0.77 ± 0.10 (n=6)	0.82 ± 0.01 (n=8)

*) from ox neurohypophyses

The quantities of calmodulin found by the radioimmunoassay and the phosphodiesterase activation method are given in Table 2.

DISCUSSION

N-Acetyl-muramyl-L-alanyl-D-isoglutamine (MDP) (14) represents the minimal structure required for substitution of mycobacteria in Freund's complete adjuvant. At the same time it is non-immunogenic. However, if it is coupled to a protein carrier, very high anticarrier titres can be obtained at the same time with high antiMDP-titres. The conjugation proceeds through the amino groups of calmodulin. Thus the native structure of the calmodulin molecule is only mildly modified. The modification is of the same kind as that obtained when calmodulin is iodinated using Bolton-Hunter reagent.

All rabbits immunized initially or for a booster using MDP-calmodulin conjugate responded by antibody formation with the exception of rabbits initially immunized using peroxidized calmodulin. The failure of MDP-conjugate to induce antibody formation in these animals may be explained by some immunosuppressive mechanism of the previous doses

of peroxidized calmodulin. Our failure to induce antibody formation in the case of injections of peroxidized calmodulin alone may be due to lower doses used for immunization than that used by van Eldik et al. (7). However, van Eldik et al. state that the amount of antigen administered should not be critical.

The highest titre of antibodies was seen in rabbit E which received 4 injections of thyroglobulin-calmodulin conjugate before the 2 injections of MDP-HPLC-calmodulin conjugate. The small number of animals in each group makes it difficult to state whether this procedure should be preferred.

An average radioimmunoassay sensitivity as we found it is about 10-300 times higher than that previously reported in the literature, with the exception of ref. (2) where a similar sensitivity was described. However, in this case a purified antibody was used. Most antisera reported do not or only partly distinguish between the Ca^{2+}-bound and the Ca^{2+}-free form of calmodulin. The antisera described in this paper require the calcium-free form for binding. In other words the immunoreactive site reacting with the antibody in the sera described changes when binding calcium to such an extent that it is not recognized by the binding sites of the antibody. It is described in the literature that the calmodulin molecule undergoes a conformational change after binding of calcium (i.e. it becomes more alfa-helical). This would imply that our antibody is directed to that part of the calmodulin molecule that undergoes the maximal conformational change. Its specificity is therefore different from that described by van Eldik et al. (7). This question, however, would need further study.

The fact that the antisera require the calcim-free form renders the assay complementary to the phosphodiesterase activation method where the calcium-bound form is active. The values for calmodulin content in brain homogenate agree well with the data in the literature. In the samples tested, the radioimmunoassay is giving the same values as the phosphodiesterase activation method.

This work confirmed the immunogenicity of N-acetyl--muramyl-L-alanyl-D-isoglutamine-calmodulin conjugate. The conjugation of poor antigens to MDP seems therefore to be a method of choice for enhancing their immunogenicity. An additional advantage is that the use of mycobacteria in the adjuvant for immunization may be omitted.

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