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Complex formation between γ-immunoglobulin and calmodulin in calcium-free conditions

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Abstract. We show that γ-immunoglobulin (IgG) binds calmodulin (CaM) in a Ca^{2+}-independent manner, with Kd value of $(1.7 \pm 0.5) \times 10^{-7}$ M. A single IgG molecule maximally bound 10 CaM molecules. The binding is to the heavy chain or Fab portion, but not the Fc portion, of the IgG molecules. Ca^{2+} greatly diminished the interaction between IgG and CaM, with $IC_{50} = 8-9$ μM. These data give a novel insight into protein-protein interactions.
Key words. γ-Immunoglobulin; calmodulin; Ca^{2+}.

Calmodulin (CaM) is a protein found in a wide variety of eukaryotic cells, tissues and organs[1]. CaM has a high affinity for Ca^{2+}, and the Ca^{2+}-CaM complex exerts regulatory functions on various cellular processes involving enzymes such as phosphodiesterase, adenylate cyclase, protein kinase, phosphatase, and Ca^{2+}-ATPase[1]. However, three neuronal proteins, neuromodulin (also called GAP-43 or B50)[2–7], neurogranin[8], and p190[9] were recently shown to bind CaM only in the absence of Ca^{2+}. Moreover, it was reported that mutant CaM that essentially lacked any affinity for Ca^{2+} supported yeast cell growth[10]. Since CaM is an abundant intracellular protein[1] and since intracellular concentration of Ca^{2+} may oscillate during cellular processes[11], these findings have indicated a novel aspect of Ca^{2+}-independent regulatory processes by CaM. In this paper, we present data which clearly show another example of a Ca^{2+}-independent CaM-protein interaction.

Materials and methods

Chemicals. IgG was obtained from commercial sources or prepared in our laboratory using a Bio-Rad (Hercules, CA, USA) IgG preparation kit. Human Fab and Fc fragments were purchased from Rockland, Pennsylvania, USA. [^{125}I]-CaM and bovine brain CaM were purchased from New England Nuclear and Calbiochem (La Jolla, CA, USA), respectively. Chicken heart lactate dehydrogenase, CaM-agarose, protein A Sepharose CL-6B and Immobilon-P membrane were the products of Wako Pure Chemicals (Osaka, Japan), Sigma (St Louis, MO, USA), Pharmacia (Piscataway, NJ, USA) and Millipore (Bedford, MA, USA), respectively.

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[^{125}I]-CaM overlay experiment. IgG (3.5 μg) was treated with Laemmli's sample buffer[12] in reducing (plus 2-mercaptoethanol) or non-reducing (minus 2-mercaptoethanol) conditions. Some samples were boiled at 100 °C for 3 min. These samples, after SDS-PAGE[12], were transferred to Immobilon-P membrane in 25 mM Tris/192 mM glycine/20% methanol at 100 V for 1 h. After soaking in 20 mM Tris-HCl (pH 7.5)/500mM NaCl, the membrane was blocked overnight at 4 °C in the same buffer containing 3% (w/v) BSA. The membrane was then cut into two pieces. One piece was incubated in 20 ml of 50 mM Tris-HCl (pH 7.5)/150 mM NaCl/3% (w/v) BSA/0.3 μm CaM/2.5 μCi [^{125}I]-CaM, containing 5 mM EGTA. The second peice was incubated in the same solution except that 1 mM $CaCl_2$ replaced EGTA. After 1–1.5 h incubation at room temperature with gentle agitation, each piece of membrane was washed three times (15–30 min) with 25 ml of the corresponding buffer with the omission of CaM. The membrane pieces were air-dried and subjected to autoradiography. Fab fragment (2 μg) or Fc fragment (2 μg) were similarly overlayed with [^{125}I] CaM using the same procedure. Experimental conditions for individual lanes are given at the bottom of figures 1 and 2.

CaM-agarose affinity chromatography. To test the specificity of the CaM-IgG interaction, we prepared a mixture of IgG (50 μg) and lactate dehydrogenase (50 μg, selected as a representative of cytosolic proteins, since CaM is found predominately in the cytosol) in 1 ml of 40 mM Tris-HCl (pH 7.5)/150 mM NaCl/1 mM EGTA, which was loaded to 0.5 ml of CaM-agarose affinity column. The pass-through fraction was reloaded to the column which was then washed with 20 ml of the same buffer. The CaM-bound protein was eluted with 40 mM Tris-HCl (pH 7.5)/150 mM NaCl/0.1 mM EGTA/1 mM $CaCl_2$. Eluates were fractionated in

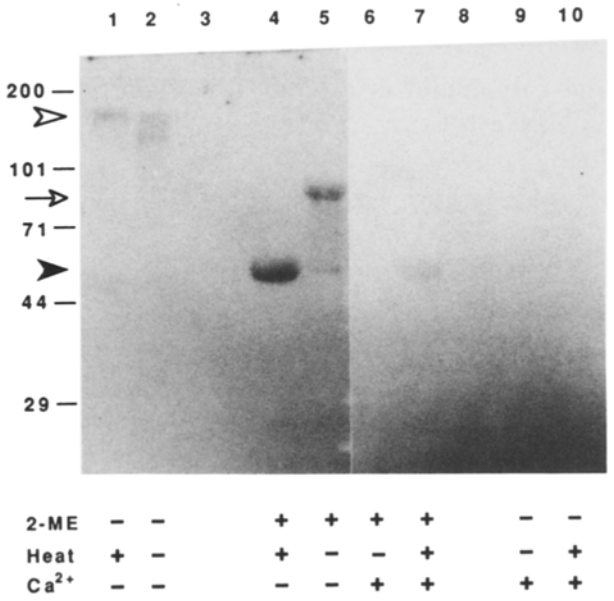

Figure 1. [^{125}I]-CaM overlay experiment with IgG. IgG, boiled or unboiled in the sample buffer, was electrophoresed under reducing or non-reducing conditions and blotted to Immobilon-P membrane that was then overlayed with [^{125}I]-CaM in the presence of EGTA or Ca^{2+}. Open arrowhead, filled arrowhead and open arrow indicate [^{125}I]-CaM binding to 150 kDa IgG molecule, 50 kDa heavy chain and 75 kDa 'single heavy chain-single light chain' complex, respectively. Various animal species such as human, rabbit, goat and rat worked equally well as sources of IgG. Lanes 3 and 8 were used to buffer the spread of reducing agent, 2-mercaptoethanol from reducing lanes 4–7 to non-reducing lanes 1, 2, 9 and 10. Based on 'plus' Ca^{2+} and 'minus' Ca^{2+} conditions of CaM overlay experiment, lanes were arranged in a mirror image of counterpart samples such as lanes 1 vs 10,5 vs 6, etc. Migration rates of prestained molecular standards (BRL) are marked in kDa on the left.

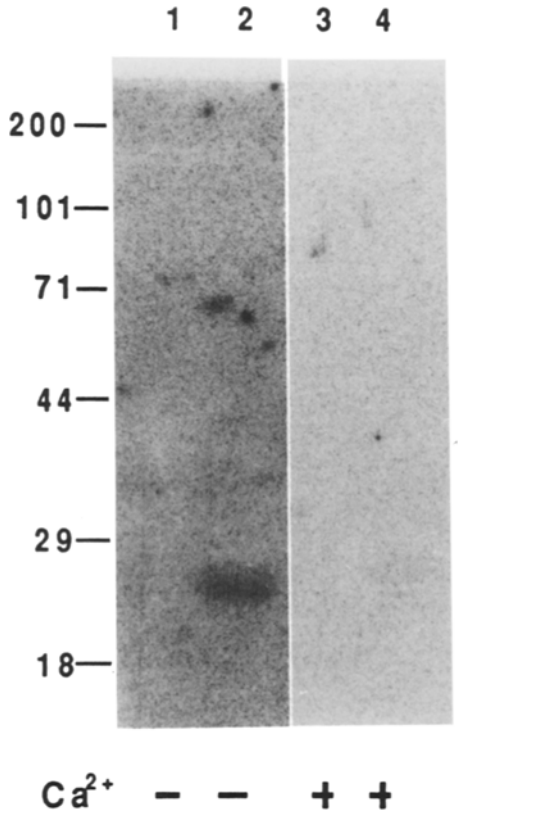

Figure 2. [^{125}I]-CaM binding to Fab fragment, but not to Fc fragment. Fab fragment (lanes 2 and 4) and Fc fragment (lanes 1 and 3) were heated under reducing conditions. SDS-PAGE, Western blotting and [^{125}I]-CaM overlay experiment were performed as in figure 1 in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of Ca^{2+}.

0.5 ml steps and then 10 µl of each fraction was analyzed by SDS-PAGE[12] under non-reducing conditions. A mixture of IgG and BSA was similarly applied to a CaM-agarose column chromatography and analyzed by SDS-PAGE.

Scatchard analysis of [^{125}I]-CaM binding to IgG. In 1.5 ml microcentrifuge tubes, human IgG (1 nM) was incubated with varying concentrations of [^{125}I]-CaM (1–100 nM, specific activity: 0.059 µCi/pmol) in 50 µl phosphate buffered saline (PBS)/0.1% BSA/4 mM EGTA. For the analysis of non-specific binding, a 100-fold excess of CaM was included. After overnight incubation at 4 °C, 80 µl suspension of protein A Sepharose CL-6B (one-tenth gram resin preswollen in 2.200 µl PBS/4 mM EGTA) and 370 µl PBS/0.1% BSA/4 mM EGTA were added. One hour later, the ternary complex of protein A/IgG/[^{125}I]-CaM was briefly spun down. The complex was washed once with 0.8 ml PBS/0.1% BSA/4 mM EGTA and twice with 0.8 ml PBS/4 mM EGTA. The radioactivity retained in the ternary complex was measured by a Cobra auto-gamma (Packard). In another experiment, [^{125}I]-CaM binding to IgG molecule in 40 mM Tris-HCl (pH 7.5)/150 Mm NaCl/0.1% BSA/50 µM EGTA plus various concentration of $CaCl_2$ was analyzed. An affinity constant, $K_{pH}^{Ca\text{-}EGTA} = 4.4 \times 10^6\ M^{-1}$ was employed to obtain the desired free Ca^{2+} concentrations[13]. The ternary complex of CaM-[^{125}I]-IgG-protein A Sepharose CL-6B was washed with the same buffer system containing the corresponding concentration of Ca^{2+}, and then the [^{125}I]-CaM-IgG complex was quantified by a Cobra auto-gamma.

Other methods. Protein content was determined by the bicinochoninic acid method[14] using a kit supplied by Pierce (Rockford, IL, USA).

Results and discussion

Figure 1 shows the result of SDS-PAGE and Western blotting of IgG followed by [^{125}I]-CaM overlay analysis. When IgG was electrophoresed under non-reducing conditions and blotted, [^{125}I]-CaM was found to bind to the 150 kDa IgG molecule in the absence of Ca^{2+}, whereas [^{125}I]-CaM binding to IgG was much less significant in the presence of Ca^{2+} (see open arrowhead and compare lanes 1 and 2 with lanes 10 and 9, respectively). When the electrophoresis was run under reducing conditions, IgG boiled in Laemmli's sample buffer

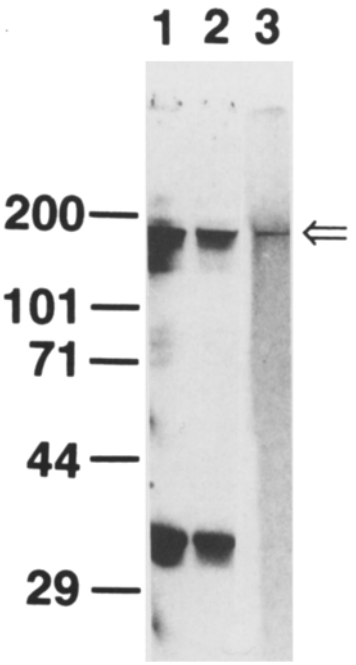

Figure 3. CaM-agarose affinity chromatography of IgG. A mixture of IgG and lactate dehydrogenase was applied to a CaM-agarose column in the absence of Ca^{2+}. After a column wash with Ca^{2+}-free buffer, the divalent cation was employed to elute the CaM-bound protein from the column. Aliquots of the protein mixture prior to the column chromatography (lane 1), CaM-agarose unbound protein (lane 2) and the first fraction of Ca^{2+} eluate (lane 3) were analyzed on SDS-PAGE under non-reducing conditions. Because of the limited binding capacity of the column, the 150 kDa IgG band of the Ca^{2+} eluate was detected only by silver staining (lane 3, arrow), while lanes 1 and 2 were stained by Coomassie brilliant blue R-250, showing a lactate dehydrogenase band.

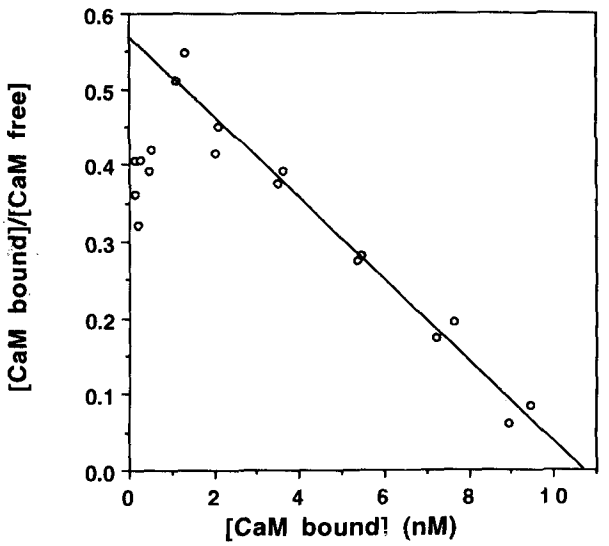

Figure 4. Scatchard analysis of [^{125}I]-CaM binding to IgG. The [^{125}I]-CaM binding to IgG was quantified as described in 'Materials and methods'. Plots were obtained after repeated experiments with duplicated samples.

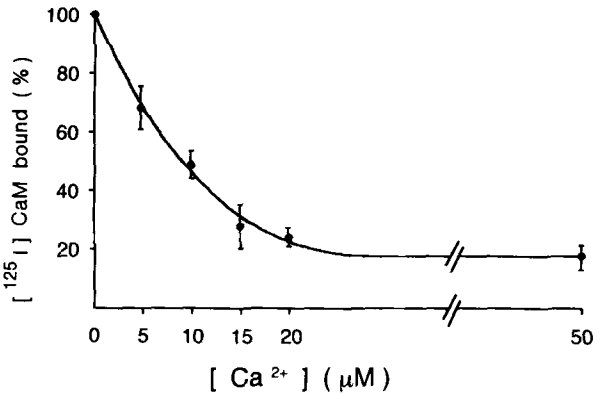

Figure 5. Ca^{2+} effect on [^{125}I]-CaM binding to IgG. [^{125}I]-CaM binding to IgG was analyzed in the presence of various concentrations of Ca^{2+}. The bound [^{125}I]-CaM was expressed as % of control assay in which no Ca^{2+} was present.

gave a 50 kDa band to which [^{125}I]-CaM was bound in the absence of Ca^{2+} (filled arrowhead, lane 4), while unboiled IgG bound predominantly to [^{125}I]-CaM a 75 kDa band in the absence of Ca^{2+} (open arrow, lane 5). Again, significantly less CaM binding was observed in the presence of Ca^{2+} (lanes 7 and 6). The 50 kDa peptide corresponds to the heavy chain of the IgG molecule. The IgG, if not boiled, was probably only partially reduced to produce a 75 kDa 'single light chain-single heavy chain' complex. The weak autoradiogram from whole IgG (lanes 1 and 2) was presumably due to poor blotting efficiency of the large-sized protein, since dot-blotted whole IgG gave a level of bound [^{125}I]-CaM similar to that of the heavy chain. No such CaM binding was observed in a similar experiment using BSA (data not shown).

The CaM-binding ability of the IgG molecule was ascribed to the Fab fragment, not to the Fc fragment as shown in figure 2 (lanes 2 and 1). The Ca^{2+}-free condition was again confirmed for the CaM binding, since low levels of signal were observed in lanes 3 and 4 where Ca^{2+} was included in the overlay assay.

We further confirmed the complex formation between IgG and CaM using CaM-agarose affinity chromatography. When a mixture of IgG and lactate dehydrogenase was applied to this affinity column, IgG was selectively trapped by the column in the absence of Ca^{2+} and was eluted from it once Ca^{2+} was present in the elution buffer, as indicated in lane 3 of figure 3 (see arrow). Replacement of lactate dehydrogenase with BSA gave similar result (data not shown).

Scatchard analysis indicated the affinity constant between IgG and CaM was $Kd = (1.7 \pm 0.5) \times 10^{-7}$ M (fig. 4). Interestingly, a single IgG molecule maximally bound approximately 10 molecules of CaM (fig. 4). Ca^{2+} strongly attenuated the CaM binding to IgG. The IC_{50} of the divalent cation was 8–9 μM under the condition tested (fig. 5).

Although we currently do not know the physiological significance of the IgG-CaM interaction, the data re-

ported here introduce a new aspect of protein-protein interaction.

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