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Calcineurin: A calcium- and calmodulin-binding protein of the nervous system

(cyclic nucleotide phosphodiesterase/neurotransmitters)

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ABSTRACT **The inhibitory protein that binds calmodulin and thus prevents activation of several Ca^{2+}-dependent enzymes by calmodulin is shown to also bind four Ca^{2+} per mol of protein with high affinity ($K_d \leq 10^{-6}$ M). On the basis of its Ca^{2+}-binding properties and its localization to nervous tissue, the inhibitory protein is now called "calcineurin." Calcineurin is composed of two subunits: calcineurin A (61,000 M_r) which interacts with calmodulin in a Ca^{2+}-dependent fashion, and calcineurin B (15,000 M_r) which binds Ca^{2+}. The interaction of calcineurin A with calcineurin B is independent of Ca^{2+} or Mg^{2+}. The dual interaction of calcineurin A with two different Ca^{2+}-binding components and the high affinity of calcineurin for Ca^{2+} suggest a possible role for calcineurin in the regulation of free Ca^{2+} concentrations in the nervous system. Calcineurin may thereby modulate the release and action of neurotransmitters.**

A heat-labile factor that inhibits the activation of cyclic nucleotide phosphodiesterase by calmodulin was described by Wang and Desai (1). Independently, the major calmodulin-binding component of brain was purified in our laboratory and shown to be the inhibitor protein (2). It was also shown to inhibit the stimulation, by calmodulin, of adenylate cyclase (3, 4), turkey gizzard myosin light chain kinase (R. S. Adelstein, D. R. Hathaway, and C. B. Klee, unpublished observations), erythrocyte Ca^{2+}, Mg^{2+}-ATPase (5, 6), and phosphorylase b kinase (7). The protein appears to be specific to the nervous system because it has not been detected in other tissues by direct assay, although very small amounts of it were detected by radioimmune assays (W. Y. Cheung, personal communication; ref. 8). The function of the inhibitory protein is unknown. We now show that this protein, which binds calmodulin by a Ca^{2+}-dependent mechanism (1–4), can itself also bind Ca^{2+} with high affinity. Previously, the inhibitor was referred to as calmodulin-binding protein (1) or inhibitory protein of cyclic nucleotide phosphodiesterase (2–4). On the basis of its Ca^{2+}-binding properties and its specificity for the nervous system we now propose to call it "calcineurin."

MATERIALS AND METHODS

Calcineurin was purified from bovine brain as described (2). Rechromatography on Sephadex G-200 was omitted because the protein obtained after the first gel filtration was devoid of cyclic nucleotide phosphodiesterase activity and contained only the two polypeptides characteristic of calcineurin (1–4). Rabbit skeletal muscle troponin C was the generous gift of Paul Leavis. Bovine brain calmodulin and calmodulin-Sepharose were prepared by published procedures (2, 9). $^{45}CaCl_2$ (2.1 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was a product of New England Nuclear; dimethylsuberimidate dihydrochloride was obtained from Pierce.

Electrophoresis under denaturing conditions was performed in 15% acrylamide or 7.5 to 15% gradients of acrylamide by the Laemmli system (10). Quantitation of polypeptide chains was by densitometer tracings of photographs of the Coomassie blue-stained gels with a Joyce Loebl microdensitometer (model 3CS) and subsequent weighing of the traced peaks. At three different protein concentrations the staining intensity was linear with respect to protein concentration.

Interaction of calcineurin with Ca^{2+} was measured at 0–4°C by the method of Hummel and Dreyer as described in ref. 11. Prior to the experiment, the protein solution was dialyzed overnight against 1000 vol of 50 mM Tris·HCl, pH 8.1/0.1 M KCl/1 mM $MgCl_2$/0.2 mM dithiothreitol (buffer A). The free Ca^{2+} concentration, determined in the dialyzate by atomic absorption spectrometry, was 0.5 μM. A column of Sephadex G-25 (0.9 × 12 cm), equilibrated with buffer A at various concentrations of $^{45}CaCl_2$ was loaded with a 0.2-ml sample of calcineurin in buffer A (0.5 mg/ml) whose $^{45}CaCl_2$ concentration was adjusted to the same total concentration as that of the column buffer. The fraction size was 0.35 ml and the flow rate 4 ml/hr. Prior to the experiment the buffer solutions were freed of contaminating Ca^{2+} by treatment with Chelex-100. Polyethylene containers were used throughout. The specific activity of $^{45}CaCl_2$ was based on Ca^{2+} concentrations determined by atomic absorption spectrometry (buffer solutions and appropriate aliquots eluted from the column including the peak and trough tubes as well as tubes 30–33 were tested for Ca^{2+} concentration). Protein concentration was determined spectrophotometrically ($\epsilon^{1\%}_{277\ nm} = 9.6$) (2) and by amino acid analysis. At high concentration of Ca^{2+} (75 μM), binding of Ca^{2+} to calcineurin was measured by equilibrium dialysis in buffer A (0–4°C, 36 hr). Protein concentration was 0.5 mg/ml. Prior to the experiment the protein was dialyzed against 500 vol of buffer A with 2 μM ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid (EGTA), followed by 500 vol of buffer A with 1 μM EDTA. Quadruplicate aliquots of dialysis fluid and protein solutions were used for the radioactivity measurements.

Crosslinking of the protein with dimethylsuberimidate according to Davies and Stark (12) was performed as described (2).

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Abbreviation: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

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† This protein was not detected in the ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid eluate of calmodulin-Sepharose columns loaded with extracts from smooth muscle, platelets, or fibroblasts (unpublished observations).

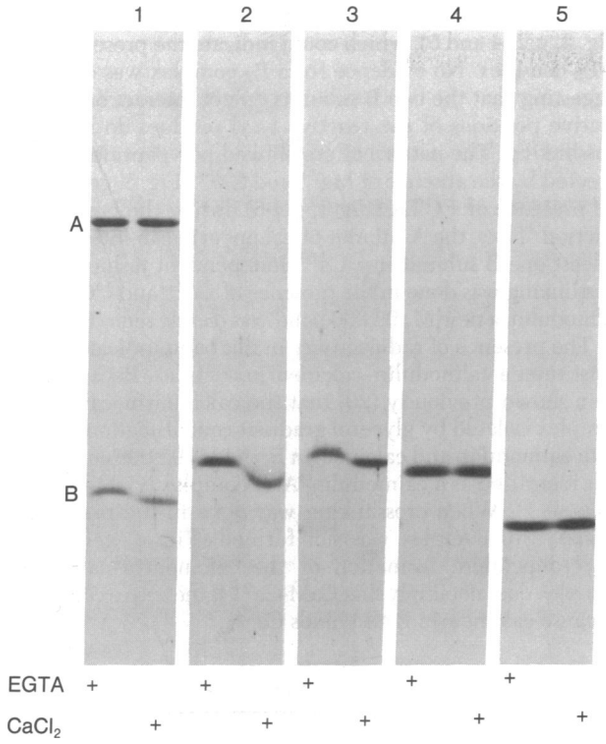


FIG. 1. Effect of Ca^{2+} and EGTA on the electrophoretic mobilities of Ca^{2+}-binding proteins. Protein samples (2 μg in 20 μl) in 50 mM Tris·HCl (pH 8.0) containing 1 mM Ca^{2+} or 1 mM EGTA were subjected to electrophoresis in the presence of sodium dodecyl sulfate in 7.5–15% gradients of acrylamide. Additions of Ca^{2+} or EGTA were as indicated. The M_r markers were bovine serum albumin, catalase, fumarase, lactate dehydrogenase, β-lactoglobulin, α-lactalbumin, and lysozyme. Columns: 1, calcineurin A (M_r 61,000) and calcineurin B (M_r 16,500 or 15,500 in the presence of EGTA or Ca^{2+}, respectively); 2, calmodulin (M_r 19,000 in EGTA or 17,000 in Ca^{2+}; 3, troponin C (M_r 19,500 in EGTA or 18,500 in Ca^{2+}; 4, β-lactoglobulin (M_r 17,500); 5, lysozyme (M_r 14,200).

RESULTS

Calcineurin, isolated in our laboratory, was shown to be composed of two polypeptide chains with M_rs of 61,000 (calcineurin A) and 15,000 (calcineurin B),‡ respectively. Upon crosslinking with dimethylsuberimidate, the two subunits were shown to form a 1:1 complex, AB (2). A similar stoichiometry has been obtained by Wallace *et al.* (4). More recently we observed that the ratio of the two subunits determined by gel electrophoresis can vary from 0.8 to 1.6 subunit B for each A subunit, depending on the purification procedure used and the extent of the purification achieved. The homogeneity of calcineurin B was confirmed by two-dimensional electrophoresis (14). Calcineurin with an AB_2 structure has been reported by Sharma *et al.* (13). The protein used in the present studies contained 1.6 B subunits and 1 A subunit as determined by Coomassie blue staining intensity, with the assumption that the staining intensities of the two subunits are identical and proportional to the M_r of the polypeptides.

Effect of Ca^{2+} on the Electrophoretic Mobility of Subunit B. As shown in Fig. 1, column 1, the electrophoretic mobility of the B subunit, but not that of the A subunit, depended on whether Ca^{2+} or EGTA was present in the sample. When 1 mM Ca^{2+} was present, the apparent M_r was 17,000; in the presence of 1 mM EGTA, the mobility was decreased and the apparent M_r was 19,000. Similar behavior was displayed by two other Ca^{2+}-binding proteins, troponin C and calmodulin (Fig. 1). The effect of Ca^{2+} on the electrophoretic mobility of calmodulin, both in the absence (15) and presence of detergent (16), has been observed previously. In contrast, the electrophoretic mobilities of β-lactoglobulin and lysozyme (Fig. 1, columns 4 and 5) and α-lactalbumin (data not shown) were not affected by Ca^{2+} or by EGTA.

‡ The terminology is that of Sharma *et al.* (13).

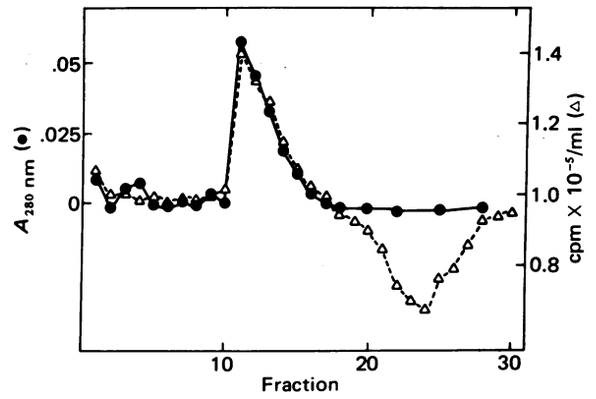


FIG. 2. Calcium binding to calcineurin. Gel filtration of calcineurin was performed in the presence of 5.4 μM $^{45}CaCl_2$ (specific activity, 10 Ci/mol). $CaCl_2$ concentration was measured directly in tubes 1, 11, 24, and 33.

EDTA was as effective as EGTA in its ability to modify the electrophoretic behavior of calmodulin. High concentrations of the chelating agents (1 mM) were required to produce the effect. No change in mobility was apparent when 0.1 mM EGTA was used. The requirement for a high concentration of chelator suggests that the electrophoretic change results from EGTA binding rather than from a conformational change induced by loss of Ca^{2+}. Another Ca^{2+}-binding protein, parvalbumin, has also been shown to interact with EGTA at millimolar concentrations (17).

Binding of Ca^{2+} to Calcineurin. Direct evidence for the binding of Ca^{2+} to calcineurin was obtained by gel filtration of the protein in the presence of several concentrations of Ca^{2+} by the method of Hummel and Dreyer (11) and by equilibrium dialysis against 75 μM $^{45}Ca^{2+}$. All the experiments were carried out in the presence of 1 mM $MgCl_2$. The protein eluted at the void volume of the column was associated with bound Ca^{2+} (Fig. 2). Binding of $^{45}Ca^{2+}$ to the protein resulted in a decrease of $^{45}Ca^{2+}$ concentration, indicated by a trough in the included volume of the column. The slight difference in the size of the peak (60,000 cpm) and that of the trough (66,000 cpm) may be due to the fact that only 75% of the protein was recovered from the column. The stoichiometry of the Ca^{2+}–calcineurin complex was determined by radioactivity and protein measurements in the peak (Table 1). Similar results were obtained

Table 1. Binding of Ca^{2+} to calcineurin

Exp.*	Ca^{2+}, μM	Bound Ca^{2+}, mol/mol† calcineurin
1	3.5 ± 0.1	3.8 ± 0.1
2	5.4 ± 0.2	3.8 ± 0.1
3	8.9 ± 0.2	3.6 ± 0.3
4	22.2 ± 1.3	4.0 ± 0.4
5	75.0 ± 9.0	6.3 ± 1.3

Data are shown as means ± SD.

* In experiments 1–4, binding was measured by the method of Hummel and Dreyer (11). In experiment 5, binding was measured by equilibrium dialysis at 0–4°C for 36 hr.

† The stoichiometry was calculated by assuming a M_r of 85,000 ($A_1B_{1.6}$).

whether the protein concentration was determined by absorption at 280 nm ($\epsilon^{1\%}_{277} = 9.6$) or by amino acid analysis (assuming an average M_r of 85,000 and a ratio of subunits A to B of 1 to 1.6). The amino acid composition of the recovered protein was not significantly different from that of the starting material, indicating that neither of the subunits was selectively lost during the gel filtration. As shown in Table 1 the extent of Ca^{2+} binding was independent of Ca^{2+} concentration when measured between 3 and 22 μM Ca^{2+}, indicating that calcineurin binds Ca^{2+} tightly ($K_d \leq 10^{-6}$ M). The larger number obtained after equilibrium dialysis against 75 μM Ca^{2+} may be due to incomplete removal of EGTA or to low-affinity Ca^{2+}-binding sites. The binding of Ca^{2+} to calcineurin in the presence of 1 mM $MgCl_2$ is therefore tighter than is the binding of Ca^{2+} to calmodulin in the presence of the same $MgCl_2$ concentration ($K_d = 2\text{–}3 \times 10^{-6}$ M) (18, 19). Calcineurin binds 4 mol of Ca^{2+} per mol. The number of Ca^{2+}-binding sites on calcineurin B is not yet clear because of the uncertainty in the number of small subunits per mol of calcineurin. Like calmodulin, calcineurin undergoes a conformational transition upon binding Ca^{2+}. UV difference spectroscopy indicated a change in the environment of both tyrosyl and tryptophanyl residues (data not shown).

Ca^{2+}-Independent Interaction of Subunits A and B. The A subunit of calcineurin is known to interact, in a Ca^{2+}-dependent fashion, with another Ca^{2+}-binding protein, calmodulin (13, 20). It was therefore important to determine whether or not the interaction of the A subunit with the B subunit was also Ca^{2+}-dependent. When the protein was crosslinked with dimethylsuberimidate the M_r 61,000 polypeptide (unreacted A subunit) disappeared after prolonged incubation and was replaced by a M_r 76,000 band (A–B complex) (Fig. 3). A faint

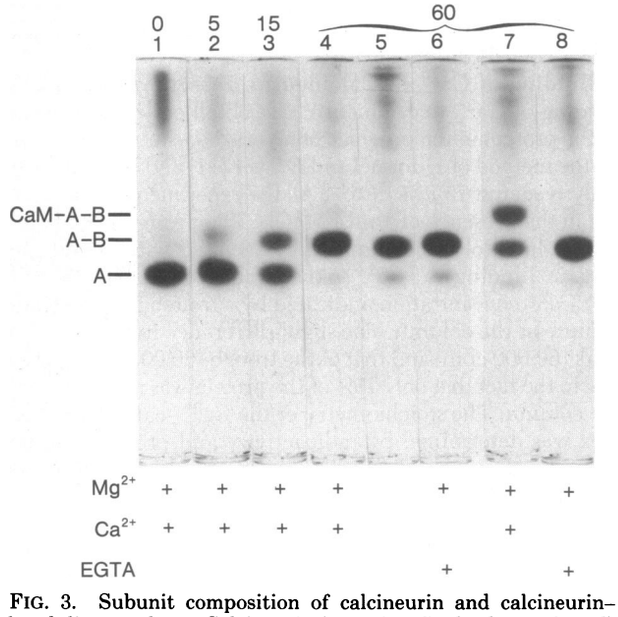
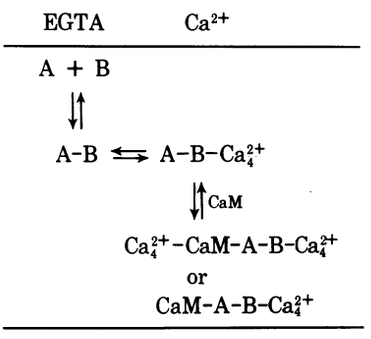


FIG. 3. Subunit composition of calcineurin and calcineurin–calmodulin complexes. Calcineurin (10 μg in a final volume of 20 μl) was incubated at 23°C for various times in the presence of dimethylsuberimidate (1 mg/ml, gels 1–6; 5 mg/ml, gels 7 and 8). When present, $MgCl_2$ was 1 mM, $CaCl_2$ was 0.5 mM, and EGTA was 1 mM. EDTA (gel 5) was 2 mM; calmodulin (gels 7 and 8) was 3 μg in 20 μl. Electrophoresis was for 10 hr to maximize the band resolution. Under these conditions, calmodulin and calcineurin B migrated out of the gels. The M_r markers were the products of crosslinking of lactate dehydrogenase and fumarase. The identity of the A–B-calmodulin complex (CaM–A–B) was confirmed by using [^{14}C]guanidinated calmodulin; radioactivity was recovered in the M_r 76,000 and 92,000 bands (gel 7). In the presence of EGTA (gel 8), no radioactivity was found in the M_r 76,000 band.

M_r 92,000 species was sometimes present after long incubation (Fig. 3, gels 4 and 5), which could indicate the presence of an A–B_2 complex. No evidence for a B_2 complex was obtained, suggesting that the two B subunits do not interact or that the relative positions of the reactive lysyl residues do not allow crosslinking. The pattern of crosslinked polypeptides was not affected by the absence of Mg^{2+} and Ca^{2+} (Fig. 3, gel 5) or by the presence of EGTA (Fig 3, gel 6) during the crosslinking reaction. Thus, the A subunit of calcineurin can interact with at least one B subunit in a Ca^{2+}-independent fashion. When crosslinking was done in the presence of Ca^{2+} and ^{14}C-labeled calmodulin, a new M_r 92,000 band was clearly seen (Fig. 3, gel 7). The presence of radioactivity in this band indicated that it constitutes a calmodulin–calcineurin complex. Because it has been shown previously (20) that the calcineurin–calmodulin complex isolated by glycerol gradient centrifugation contains both calmodulin and calcineurin B, the M_r 92,000 component was identified as a calmodulin–A–B complex (CaM–A–B; see scheme 1). When crosslinking was done in the presence of EGTA, this complex was not formed (Fig. 3, gel 8). The Ca^{2+}-dependent formation of the calcineurin–calmodulin complex does not imply that CaM-Ca^{2+} is the active component because calcineurin B also binds Ca^{2+}.



The experiments described above indicate the formation of a ternary complex involving calcineurin A, calcineurin B, and calmodulin in equimolar concentrations. Calcineurin B does not interact with itself or with calmodulin because no M_r 30,000 complex was detected on the gels. The small amount of M_r 92,000 complex detected after crosslinking in the absence of calmodulin suggests that calcineurin A may interact with a second molecule of calcineurin B, possibly at the calmodulin-binding site. However, in the absence of definitive evidence of an A–B_2 or CaM–A–B_2 complex after crosslinking experiments, calcineurin was considered to be a 1:1 complex of its two subunits (scheme 1). Calcineurin A thus interacts with two Ca^{2+}-binding proteins: calcineurin B in the absence of Ca^{2+} and calmodulin in the presence of Ca^{2+}. Interestingly, phosphorylase b kinase has recently been shown to interact with calmodulin by both a Ca^{2+}-independent and a Ca^{2+}-dependent mechanism (21).

DISCUSSION

Calcineurin, a protein specific to the nervous system (8), was isolated on the basis of its ability to inhibit phosphodiesterase (1) or to bind calmodulin (2–4, 13). We demonstrate here that calcineurin is also a Ca^{2+}-binding protein with a high affinity for Ca^{2+} ($K_d \leq 10^{-6}$ M) in the presence of physiological concentrations of Mg^{2+}. These properties suggest a role for calcineurin in the control of Ca^{2+}-dependent processes in the brain. Ca^{2+} plays a critical role in several neuronal processes, including the biosynthesis (22) and release of neurotransmitters

at the synaptic terminals (23). There is strong evidence that Ca^{2+} influx, induced by depolarization of the terminal, triggers the rapid release of neurotransmitters from synaptic vesicles into the synaptic cleft. Facilitation and post-tetanic potentiation of transmitter release are also believed to be Ca^{2+}-dependent phenomena (24, 25) and have been explained by a temporary increase of Ca^{2+} levels within the terminals after an action potential or a train of action potentials (26). Blaustein *et al.* (26) have postulated the existence of a nonmitochondrial Ca^{2+}-storage system with a high affinity and low capacity for Ca^{2+} which could play an important role in buffering the Ca^{2+} that enters the terminal after nerve depolarization. Calcineurin, with a high affinity for Ca^{2+} even in the presence of physiological concentrations of Mg^{2+}, is present in brain at concentrations [(1 μmol/kg) (13)] likely to be required for such a putative Ca^{2+}-storage system. The ability of calcineurin to interact with another Ca^{2+}-binding protein, calmodulin, may significantly increase the regulatory capabilities of this system. The role of calcineurin in the postsynaptic terminal is more difficult to evaluate. Although the involvement of Ca^{2+} in postsynaptic processes has not been clearly defined, several enzymes under the control of Ca^{2+} and calmodulin, including adenylate cyclase, cyclic nucleotide phosphodiesterase, and Ca^{2+}-dependent kinases and their substrates are localized at this level (27, 28). Wood *et al.* (29) have recently shown, by immunofluorescence techniques, that calmodulin and calcineurin are present in the postsynaptic membranes, and Grab *et al.* (16) have also shown the presence of calmodulin in isolated postsynaptic densities. We have reported (30) that calcineurin binds to calmodulin more tightly than does phosphodiesterase but that cyclic nucleotides increase the affinity of phosphodiesterase for calmodulin. It is therefore possible that calcineurin acts as a calmodulin buffer. The increase in cyclic AMP levels resulting from the stimulation of adenylate cyclase could activate phosphodiesterase by translocating calmodulin from calcineurin to phosphodiesterase. Activation of phosphodiesterase would decrease cyclic nucleotide levels to those characteristic of the resting state. Precise information on the interactions of calmodulin, calcineurin, and other proteins under Ca^{2+} control may help to elucidate this complex regulatory mechanism.

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