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IDENTIFICATION OF NOVEL CALCIUM BINDING PROTEINS OF HEART AND BRAIN 100,000 X G SUPERNATANT

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Summary: The chelex competitive calcium binding assay has been used to assay the calcium binding activity of the 100,000 x g supernatant of bovine heart and brain. Chromatography of brain 100,000 x g supernatant on diethylaminoethyl (DEAE) cellulose reveals the presence of two peaks of calcium binding activity, peak I eluting at about 0.05 M NaCl and peak II at about 0.18 M NaCl. Chromatography of peak I on Sephadex G-150 resolves a major and a minor peak of calcium binding activity, at M_r 40,000 and M_r 150,000. Chromatography of peak II (0.18 M NaCl) on Sepharose 6B produces two peaks of calcium binding activity, a broad peak of calcium binding activity composed of two molecular weight species of M_r 230,000 and M_r 420,000, and a sharp peak of calcium binding activity with M_r 75,000. Chromatography of the 100,000 x g supernatant of bovine heart on DEAE Cellulose reveals two peaks of calcium binding activity. Chromatography of the lower ionic strength peak on Sephadex G-150 resolved major and minor peaks of calcium binding activity at M_r 65,000 and 150,000, respectively. The results of this study suggest the presence of several calcium binding proteins, other than calmodulin, in these tissues.

The calcium binding proteins play a fundamental role in the calcium second messenger system; they serve to transduce the calcium second message into cellular activation (1-3). Several cytosolic calcium binding proteins have been reported (reviewed in 4, 5) but of these proteins only calmodulin has been shown to play a fundamental role in the calcium second messenger system. Recently, several investigators have suggested that calmodulin is the primary cytosolic calcium receptor (6,7), although this theory has not been directly tested.

In the present report we have chromatographed the 100,000 x g supernatant of bovine heart and brain on DEAE Cellulose and compared the calcium binding activity with the calmodulin activity. In both tissues the majority of the calcium

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binding activity eluted in peaks distinct from the calmodulin activity. This result suggests that in heart and brain, calmodulin may not be the primary cytosolic calcium receptor.

METHODS AND MATERIALS

$^{45}CaCl_2$ was purchased from Amersham. Chelex 100 was purchased from BioRad. DEAE Cellulose (DE 52) was purchased from Whatman. All chemicals were of reagent grade or better. Calmodulin was assayed on the basis of its ability to activate the calcium activatable phosphodiesterase of bovine brain (9). Routinely, aliquots were incubated at 98°C for two minutes before assaying calmodulin. Protein concentration was determined according to Bradford (10) using bovine serum albumin as standard.

Calcium binding activity was measured by the chelex competitive calcium binding assay (8,11,12). Chelex 100 (minus 400 mesh) was equilibrated with 100 mM Tris (pH 7.5) and 50 mM NaCl in an approximate ratio of 1:10 (chelex/buffer). To a final reaction mixture volume of 1.0 ml was added variable concentrations of test substance, $^{45}CaCl_2$ (0.1-0.25 μCi/ml), and 0.025 ml of rapidly stirring Chelex 100. The mixture was incubated with continual agitation at room temperature in Eppendorf plastic tubes. After twenty minutes, the tubes were centrifuged at 2,000 x g for two minutes and 0.1 ml aliquots of supernatant were removed. Radioactivity was determined by liquid scintillation spectrometry. A control sample of chelex and reaction media without test substance was included in each assay. The results of the chelex assay are expressed as "% Total Counts" where % Total Counts = (cpm in test - cpm in control) X 100/total cpm added. Typically, the control contained 2-4% of total cpm added. The assay has been described in details elsewhere (8).

Fresh bovine hearts and brains were obtained from a local slaughterhouse. Connective tissue was dissected away and the organs were rinsed thoroughly in ice cold distilled water and frozen immediately. Five hundred grams of frozen tissue was chopped and then minced in a meat grinder. The mince was mixed with three volumes (weight/volume) of ice cold 40 mM Tris/HCl (pH 7.5) containing phenylmethylsulfonylchloride (1.0 mM), diisopropylfluorophosphate (1.0 mM), 0.2 mM EDTA, and homogenized in a Waring blender. The resultant crude fraction was centrifuged at 20,000 x g for thirty minutes and the supernatant centrifuged for seventy-five minutes at 100,000 x g. The 100,000 x g supernatant was diluted into five volumes of 10 mM Tris (pH 7.5) and 800 ml of packed DEAE Cellulose was added. All experimental manipulations were performed at 4°C.

The mixture was stirred rapidly for one hour then filtered through a scintered glass funnel (coarse size). The resultant slurry was washed with 6.0 liters of 10 mM Tris (pH 7.5). A 5.0 x 60 cm column was poured. Protein was eluted with a linear gradient made from 2.2 liters each of 10 mM Tris (pH 7.5) and 10 mM Tris (pH 7.5), 0.45 M NaCl. Fractions (20 ml) were assayed for calcium binding activity.

RESULTS AND DISCUSSION

Several investigators have suggested that calmodulin is the primary cytosolic calcium receptor (6,7). To test this hypothesis, we have fractionated the 100,000 x g supernatant of bovine heart and brain on DEAE Cellulose and compared the calcium binding activity, assayed by the chelex competitive calcium binding assay (8), with the calmodulin activity, assayed by activation of cyclic nucleotide phosphodiesterase (9). If calmodulin is the major intracellular receptor for calcium

then a correlation between calcium binding activity and calmodulin activity would be expected.

In Figure 1, the 100,000 x g supernatant of bovine brain was chromatographed on DEAE Cellulose. Two peaks of calcium binding activity were resolved, peak I at 0.04 M NaCl and Peak II at 0.18 M NaCl. Calmodulin activity was eluted at about 0.28 M NaCl. The observation that the majority of calcium binding activity eluted as two peaks of activity distinct from the calmodulin activity appears contradictory to the suggestion that calmodulin is the primary intracellular calcium receptor (6,7). Most importantly, this result indicates the presence of calcium binding substances distinct from calmodulin in brain 100,000 x g supernatant.

In order to examine the molecular weight species responsible for calcium binding activity, peak I was concentrated by ultrafiltration (PM 10 membrane, Amicon), and applied to a Sephadex G-100 column. The results, presented in Figure 2, indicate that peak I is composed of a major peak of calcium binding activity (M_r 40,000) and a minor peak of calcium binding activity (M_r 150,000). Similarly, peak II was concentrated (PM 10) and applied to a column of Sepharose 6B. Results are presented in Figure 3. Essentially, two peaks of calcium binding activity were eluted, the first peak consisted of two poorly resolved activities at M_r 420,000 and M_r

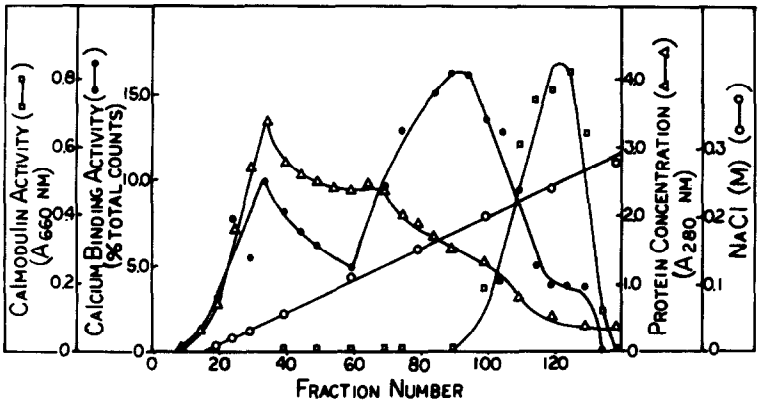

Figure 1. DEAE Cellulose chromatography of the 100,000 x g supernatant of bovine brain. The 100,000 x g supernatant was prepared from 500 grams of bovine brain, and equilibrated batchwise with 800 ml of packed DEAE Cellulose as outlined in "Methods and Materials". A 5.0 x 60 cm column was poured and developed with a linear salt gradient. Twenty ml fractions were collected and 0.5 ml of selected fractions were analysed for calcium binding activity.

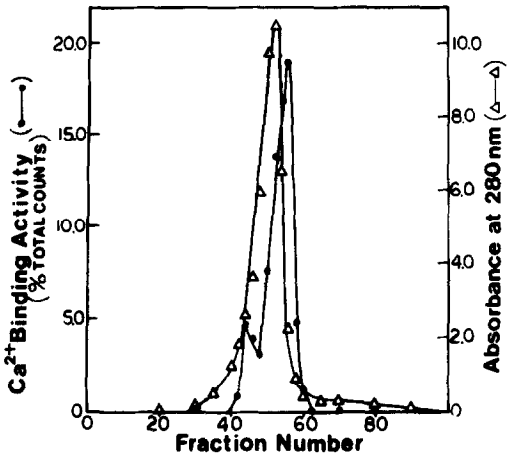

Figure 2. Chromatography of bovine brain peak I on Sephadex G-150. Peak I (Figure 1) was concentrated by ultrafiltration (PM 10, Amicon) and applied to a 2.5 x 100 cm column of Sephadex G-150. 2.0 ml fractions were collected and 0.2 ml of selected fractions were used to determine calcium binding activity.

230,000. The second peak of calcium binding activity eluted from Sepharose 6B column at about M_4 75,000.

The calcium binding activity and calmodulin activity of bovine heart 100,000 x g supernatant has also been compared. Chromatography of the 100,000 x g supernatant of bovine heart on DEAE Cellulose (Figure 4) resolves two peaks of calcium binding activity. Peak I was eluted at 0.225 M NaCl and peak II was eluted at

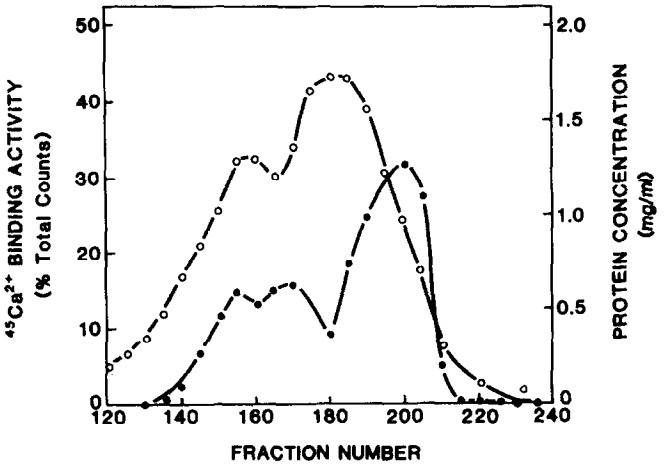

Figure 3. Chromatography of bovine brain peak II on Sepharose 6B. Peak II (Figure 2) was concentrated by ultrafiltration (PM 10, Amicon) and applied to a 2.5 x 100 cm column of Sepharose 6B. 2.0 ml fractions were collected and 0.2 ml of selected fractions were used to determine calcium binding activity. (●—●), calcium binding activity; (○—○), protein concentration.

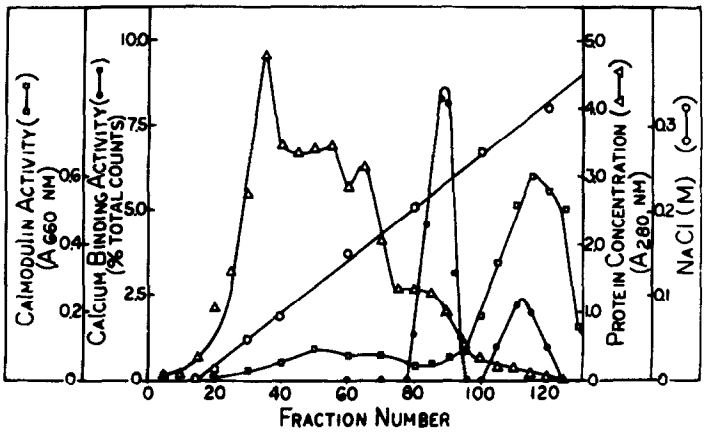

Figure 4. DEAE Cellulose chromatography of the 100,000 x g supernatant of bovine heart. The 100,000 x g supernatant was prepared from 500 grams of bovine heart, equilibrated batchwise with 800 ml of packed DEAE Cellulose, and a 5.0 x 60 cm column poured as outlined in "Methods and Materials". The column was developed with a linear salt gradient, 20.0 ml fractions were collected and 0.5 ml of selected fractions were analysed for calcium binding activity.

0.3 M NaCl. Calmodulin activity eluted at about 0.3 M NaCl. These results obtained from bovine heart (Figure 4) appear to confirm the results obtained from bovine brain (Figure 1) in that the majority of calcium binding activity does not co-elute with the calmodulin activity. In contrast to the results reported for bovine brain, in bovine heart a peak of calcium binding activity co-elutes with the calmodulin activity. In fact, subsequent purification of peak II of bovine heart (Figure 4) reveals that the calcium binding activity of peak II is due entirely to calmodulin (data now shown). Concentration of peak I (PM 10) of bovine heart and chromatography of this material on Sephadex G-150 resolves two peaks of calcium binding activity (Figure 5), at M_r 65,000 and 150,000. Therefore, the 100,000 x g supernatant of bovine heart contains two calcium binding proteins in addition to calmodulin.

In the present report the calcium binding activity of the 100,000 x g supernatant of bovine brain and liver has been compared with the calmodulin activity of these tissues. Chromatography of the 100,000 x g supernatant of brain (Figure 1) and heart (Figure 4) on DEAE Cellulose indicates that the majority of calcium binding activity does not co-elute with the calmodulin activity. Gel filtration chromatography of the calcium binding activity eluted from DEAE Cellulose has indicated the presence of calcium binding proteins, other than calmodulin, in these

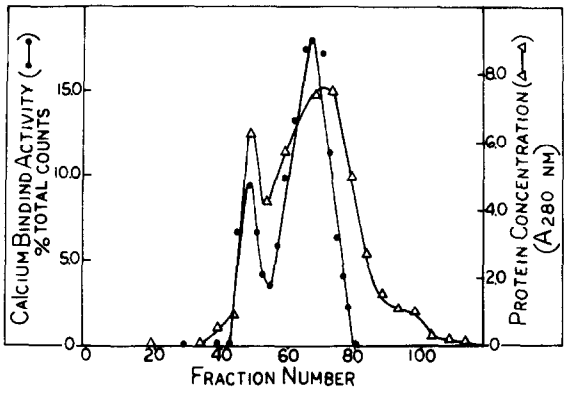

Figure 5. Chromatography of bovine heart peak I on Sephadex G-150. Peak I (Figure 4) was concentrated (PM 10, Amicon) and applied to a 2.5 x 100 cm column of Sephadex G-150. Two ml fractions were collected and 0.2 ml of selected fractions were analysed for calcium binding activity.

tissues. Similar results have been observed in bovine liver (unpublished observations).

When evaluating the possible role of a calcium binding protein in cellular calcium homeostasis, one must consider the specificity, capacity, and affinity of calcium binding as well as the concentration of protein in the tissue. The elucidation of these properties will be accomplished after purification of the calcium binding proteins. Whether the calcium binding proteins of bovine brain and heart 100,000 x g supernatant function as a calcium dependent regulatory protein similar to calmodulin, as calcium buffering proteins analogous to calsequestrin (13), or as calcium regulated enzymes, analogous to pyruvate dehydrogenase (14) also remains to be determined. The results of the present study do suggest the presence in the 100,000 x g supernatant of bovine brain and heart of several calcium binding proteins other than calmodulin. The identification of the total spectrum of calcium binding proteins of various tissues is necessary since a knowledge of all potential calcium receptor proteins is an essential part of a complete understanding of the diverse role of calcium in mediating various cellular functions.

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