Identification of a novel calcium binding protein from bovine brain

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A novel Ca^{2+} binding protein, named caligulin, was extracted from the heat-treated 100000 × *g* supernatant of bovine brain and purified to electrophoretic homogeneity. The apparent M_r of caligulin determined on sodium dodecyl sulfate polyacrylamide gels was 24000. Analysis by gel filtration chromatography indicated an apparent M_r of 33000, suggesting a monomeric protein. Amino acid composition data demonstrated the presence of 25% acidic residues, 12% basic residues and 10% leucine. In the presence of 1 mM $MgCl_2$ and 0.15 M KCl, caligulin bound 1 mol Ca^{2+}/mol protein with half-maximal binding at about 0.2 μM Ca^{2+}.

Ca^{2+} binding protein *Brain* *Caligulin* *Ca^{2+} homeostasis*

1. INTRODUCTION

The intracellular Ca^{2+} binding proteins play a fundamental role in the calcium homeostatic mechanism. Certain of these proteins such as calmodulin [1–3] serve as calcium dependent regulatory proteins and transduce the Ca^{2+} second message into cellular activation. Other Ca^{2+} binding proteins such as calsequestrin [4] may serve as Ca^{2+} buffer proteins. Still others such as mitochondrial NAD(+) dependent isocitrate dehydrogenase [5] function as Ca^{2+}-activated enzymes.

Of the known calcium binding proteins calmodulin has received the most attention in the literature. This heat-stable protein has been suggested to be the primary intracellular receptor for calcium [6]. In this report, we have fractionated the calcium binding activity of a heat-treated 100000 × g supernatant of bovine brain on DEAE-cellulose and purified a novel calcium binding activity. Results suggest that the major calcium binding protein fractionated by this procedure is not calmodulin but a novel Ca^{2+} binding protein which has been named caligulin.

Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; DTT, dithioerythritol; SDS, sodium dodecyl sulfate

2. MATERIALS AND METHODS

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed as in [7]. Protein concentration was determined as in [8].

2.1. *Protein preparation*

Bovine brain was dissected free of connective tissues, rinsed in distilled H_2O and frozen immediately at −80°C before use. 1.0 kg of frozen tissue was chopped, and homogenized in a Waring blender with 5 vol. of ice-cold 40 mM Tris (pH 7.5), 1 mM diisopropyl fluorophosphate, 0.5 mM phenylmethylsulfonyl fluoride, 1.0 mM dithioerythritol (DTT), and 20.0 ml of packed chelex-100. The resultant crude fraction was centrifuged at 10000 × *g* for 20 min and the supernatant centrifuged for 60 min at 100000 × *g*. The 100000 × *g* supernatant was then incubated at 100°C for 5 min, cooled on ice and centrifuged at 10000 × *g* for 30 min. The heat-treated 100000 × *g* superna-

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tant of bovine brain was diluted into 5 vols of 10 mM Tris (pH 7.5) to which was added 700 ml of packed DEAE-cellulose (previously equilibrated with 10 mM Tris (pH 7.5). The mixture was stirred for 1 h, filtered through a scintered glass funnel (coarse), and the resultant slurry was washed extensively with 10 l of 10 mM Tris (pH 7.5), 0.2 mM DTT. A 5.0 × 60 cm column was poured. The column was developed with a linear salt gradient made from 2.2 l each of 10 mM Tris (pH 7.5), 0.2 mM DTT and 10 mM Tris (pH 7.5), 0.2 mM DTT, 0.45 M NaCl. 20-ml fractions were collected and assayed for calcium binding activity.

2.2. *Assay of calcium binding activity*

Calcium binding activity was measured by the chelex competitive Ca^{2+} binding assay as in [9]. Briefly, 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 were 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 then incubated with continued agitation at room temperature in Eppendorf plastic tubes. After 20 min the tubes were centrifuged at 2000 × *g* for 2 min and 0.1 ml aliquots of supernatant were removed. Radioactivity was determined by liquid scintillation spectrophotometry. A control sample of chelex and reaction media without test substance was included in each assay.

2.3. *Calcium binding*

Calcium binding was determined by equilibrium dialysis. Dialysis tubing containing 0.5 ml caligulin (0.63 mg/ml) was dialysed against 2 l of buffer A [20 mM Mops (pH 7.1), 0.15 M KCl, 0.1 mM EGTA] plus 0.1 mM EDTA overnight followed by dialysis against two changes of buffer A. Each dialysis tubing was placed in 100 ml of buffer A to which was added $CaCl_2$, $^{45}Ca^{2+}$ (20 μCi), and 3.0 mM $MgCl_2$. The flasks were gently agitated for 48 h at 4°C. The protein concentration inside the dialysis tubing was determined by assuming an extinction coefficient $E^{1\%}_{280}$ of 6.184 for caligulin, determined by amino acid analysis. The free Ca^{2+} was calculated assuming a pK_d (Ca^{2+}) of 10.955 [10] for EGTA.

3. RESULTS

3.1. *Purification of heat-stable calcium binding activity*

In fig.1, the heat-treated 100000 × *g* supernatant of bovine brain was chromatographed on DEAE-cellulose. Three peaks of calcium binding activity were determined. Peak II was concentrated by ultrafiltration (PM-10, Amicon) and dialysed overnight against 20 mM Mops (pH 7.1), 0.2 mM DTT. The protein was applied to a 2.15 × 30 cm column of UltraPac TSK-545 DEAE equilibrated with 20 mM Mops (pH 7.1), 0.2 mM DTT and after a 10 bed volume wash the column was developed with a linear salt gradient. Results are presented in fig.2. Two peaks of calcium binding activity were eluted from the DEAE column, one at 0.135 M NaCl and the other at 0.150 M NaCl. Both peaks of calcium binding activity were pooled individually, concentrated and each was applied to a TSK-G 2000 SWG column. Both calcium binding activities eluted with an identical M_r 32000 (not shown) and SDS-polyacrylamide gel electrophoresis of the two proteins suggested that both proteins were of identical molecular weight.

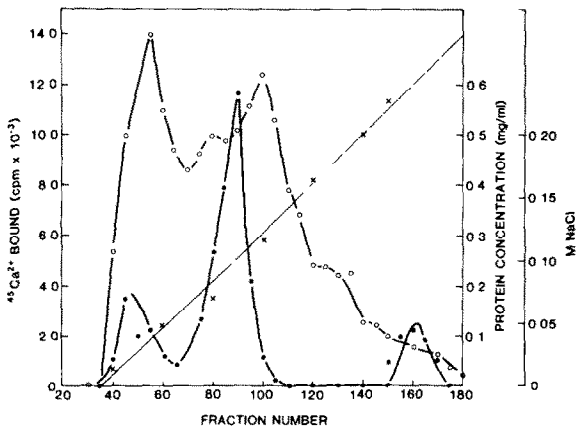

Fig.1. DEAE-cellulose chromatography of the heat-treated 100000 × *g* supernatant of bovine brain. The 100000 × *g* supernatant was prepared from 1 kg of bovine brain and equilibrated batchwise with 800 ml of packed DEAE-cellulose as outlined in section 2. A 5.0 × 60 cm column was poured and developed with a linear salt gradient. 20-ml fractions were collected and 0.5 ml of selected fractions was analysed for calcium binding activity. (●—●) Calcium binding activity; (○—○) protein concentration.

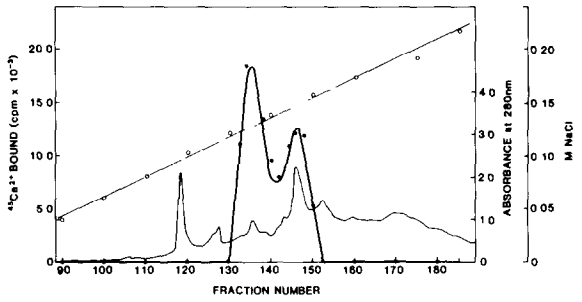

Fig.2. Chromatography of peak II by HPLC ion exchange. Peak II eluted from DEAE-cellulose (fig.1) was concentrated by ultrafiltration (PM-10, Amicon), dialysed against 20 mM Mops (pH 7.1), 0.2 mM DTT and applied to a 2.15 × 30 cm column of UltraPak TSK-545 DEAE (LKB) previously equilibrated with 20 mM Mops (pH 7.1), 0.2 mM DTT. The column was washed with 10 bed volumes and developed with a linear salt gradient made from 300 ml each of 20 mM Mops (pH 7.1), 0.2 mM DTT and 20 mM Mops (pH 7.1), 0.2 mM DTT, 0.5 M NaCl. Fractions of 2.0 ml were collected and 0.2 ml of selected fractions were assayed for calcium binding activity (●—●) and protein concentration (○—○).

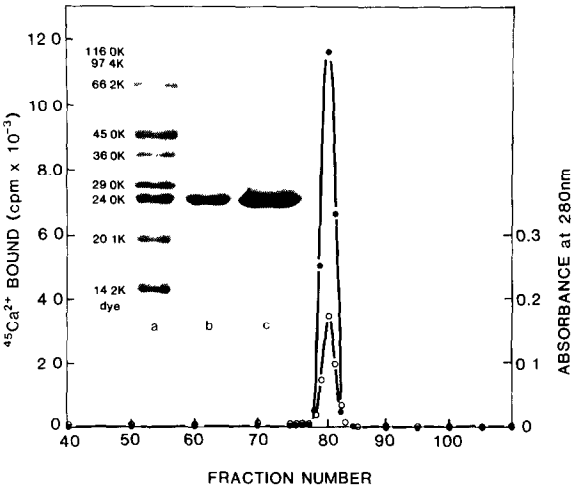

Fig.3. Chromatography of caligulin on TSK-G 2000 SWG. Caligulin was applied to a column of TSK-G 2000 SWG (LKB) which has been previously equilibrated with 20 mM Mops (pH 7.1), 0.15 M NaCl, and 0.2 mM DTT. 2-ml fractions were collected and 0.50-ml aliquots of selected fractions were assayed for calcium binding activity. (●—●) Calcium binding activity; (○—○) absorbance at 280 nm. Inset: electrophoresis of caligulin on 15% polyacrylamide gels in the presence of SDS. (a) Molecular weight standards; (b) 7.5 μg caligulin; (c) 17.5 μg caligulin. K, kDa.

However, because of the extremely low yield of the calcium binding protein eluting at 0.150 M NaCl (fig.2), further characterization was not attempted. The peak of calcium binding activity eluted from TSK-545 DEAE (fig.2) at 0.135 M NaCl was concentrated and reapplied a second time to a column of TSK-G 2000 SWG previously equilibrated with 20 mM Mops (pH 7.1), 0.15 M NaCl, and 0.2 mM DTT. As shown in fig.3, protein concentration correlated with calcium binding activity, therefore suggesting homogeneity. An M_r 32000 was determined for the calcium binding peak. Analysis on SDS-polyacrylamide gel electrophoresis (fig.3, inset) also suggests that the calcium binding protein, named caligulin, was homogeneous. An M_r 24000 was determined on SDS gels for caligulin. From 1.0 kg of bovine brain about 10.0 mg of caligulin was purified.

Table 1

Amino acid composition of caligulin from bovine brain

Amino acid	mol (%)	Residues/mol[a]
Aspartic acid	10.4	22
Threonine	4.7	10
Serine	8.6	18
Glutamic acid	14.1	30
Proline	5.3	11
Glycine	9.0	19
Alanine	6.1	13
Half-cystine[b]	1.8	4
Valine	2.4	5
Methionine	1.9	4
Isoleucine	2.7	6
Leucine	10.3	22
Tyrosine	2.8	6
Phenylalanine	7.5	16
Histidine	1.7	4
Lysine	7.5	16
Arginine	2.9	6
Tryptophan[c]	0.6	1

[a] Residues per polypeptide chain assuming an M_r of 24000 derived from SDS gels. Values are rounded off to the nearest whole number. Values presented are 24 h hydrolysis data

[b] Determined as cysteic acid after performic acid oxidation [11]

[c] Determined after hydrolysis in methanesulfonic acid at 110°C for 22 h in vacuo [12]

3.2. *Characterization of caligulin*

The amino acid composition of the 24 h acid hydrolysate of caligulin is presented in table 1. The protein is highly acidic, containing about 25% aspartic and glutamic residues. Caligulin also contains 12% basic residues and about 10% leucine.

The ultraviolet absorption spectrum of caligulin is presented in fig.4. The absorption maximum at 280 nm is common of most globular proteins and a 290 nm shoulder suggests the presence of tryptophan in the molecule (table 1). The variable region between 250 and 270 nm is consistent with the presence of a high phenylalanine : tyrosine ratio (table 1). This variable region in the ultraviolet spectrum is uncommon among most globular proteins but is demonstrated by several of the tryptophan-containing members of the calmodulin superfamily of calcium binding proteins [3].

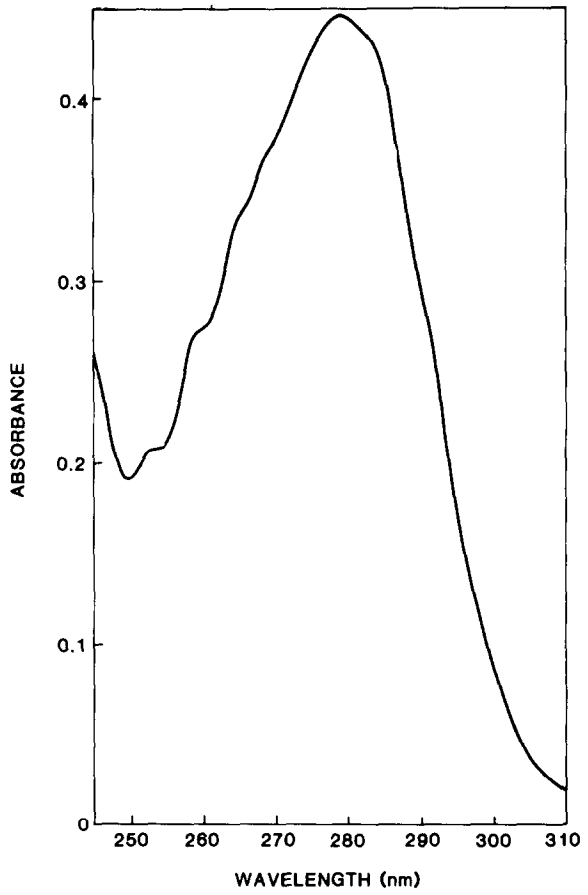

Fig.4. Ultraviolet absorption spectrum of caligulin. Caligulin concentration was 0.72 mg/ml in a pH 7.5 buffer containing 20 mM Mops, 0.1 M NaCl, 0.2 mM DTT and 0.1 mM EGTA.

Table 2

Calcium binding properties of bovine brain caligulin[a]

Free Ca^{2+}[b] (μM)	mol Ca^{2+}/mol protein[c]
0.051	0.15
0.10	0.28
0.41	0.66
0.83	0.95
1.06	0.92
50.00	0.88

[a] Equilibrium dialysis was performed in the presence of 20 mM Mops (pH 7.1), 0.15 M KCl, 3.0 mM $MgCl_2$, 0.1 mM EGTA

[b] Free Ca^{2+} was calculated assuming a pK_d (Ca^{2+}) of 10.955 [10] for EGTA

[c] The stoichiometry was calculated assuming an M_r of 24000 derived from SDS gels

The binding of Ca^{2+} to caligulin is described in table 2. In the presence of 3.0 mM $MgCl_2$ and 0.15 M KCl, caligulin bound 1 mol Ca^{2+}/mol protein, half-maximal binding occurring at 0.2 μM.

4. DISCUSSION

The results here suggest the existence in bovine brain of a novel calcium binding protein of M_r 24000 (derived from SDS gels) capable of high-affinity, specific Ca^{2+} binding.

The method of purification of caligulin is simple and straightforward. The yield of the protein can vary considerably from preparation to preparation. This is due to the association of caligulin with the pellet of the homogenate supernatant (20000 × *g* pellet of the homogenized tissue). Small variations in the time of homogenization and the amount of chelex added to the homogenization buffer dramatically influence the distribution of caligulin in the homogenate supernatant pellet. These conditions must therefore be carefully controlled. Caligulin trapped in the 200000 × *g* pellet can be easily obtained by rapidly stirring the pellet in Tris buffer (pH 7.5) containing 0.5 M NaCl and chelex-100.

The data of fig.2 show that the peak of calcium binding activity obtained from the chromatography of the 100000 × *g* supernatant on

DEAE-cellulose is eluted on TSK-545 DEAE as two peaks of calcium binding activity. Analysis of both peaks of calcium binding activity after subsequent purification, by SDS gel electrophoresis, gel filtration, and isoelectric focussing has been unsuccessful in demonstrating any difference between these peaks of activity. Furthermore, rechromatography of the proteins purified from each peak of activity (fig.2) on TSK-545 DEAE produces a single protein peak (not shown). These results suggest that the two peaks of calcium binding activity reported in fig.2 may be due to the presence of free caligulin and caligulin bound to an additional protein.

The yield of caligulin (10 mg/kg tissue) appears to be at least one order of magnitude lower than that of calmodulin. Furthermore, preliminary results have suggested that unlike calmodulin, caligulin may be present only in brain tissue. A similarity between the calmodulin superfamily of calcium binding proteins and caligulin is suggested by the ultraviolet spectrum of these proteins since caligulin and several tryptophan-containing members of this family demonstrate variable regions between 250 and 270 nm. Whether or not caligulin is actually related to this superfamily awaits further studies.

A possible physiological role for caligulin in the mediation of second messenger calcium is suggested by the calcium binding properties of this protein. In the presence of 3.0 mM $MgCl_2$ and 0.15 M NaCl, caligulin binds 1.0 mol Ca^{2+}/mol protein, half-maximal binding occurring at 0.2 μM (table 2). This study identifies caligulin as a protein capable of high affinity, and specific calcium binding.

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