Isolation of Mammalian Calelectrins: A New Class of Ubiquitous Ca^{2+}-Regulated Proteins†

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ABSTRACT: In a new approach to isolating proteins which participate in the Ca^{2+}-dependent regulation of membrane traffic in animal cells, two new Ca^{2+}-binding proteins (M_r 67 000 and 32 500) have been identified in and purified from bovine liver, brain, and adrenal medulla. These proteins specifically and reversibly bind to chromaffin granule membranes at low Ca^{2+} concentrations (half-maximal binding at 5.5 μM Ca^{2+}) and greatly potentiate the Ca^{2+}-induced aggregation of these membranes at higher concentrations (above 10 μM). In the presence of ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetate, the isolated proteins have Stokes radii of 3.40 nm (M_r 67 000) and 2.53 nm (M_r 32 500) as estimated by gel filtration and therefore occur as monomers. They are slightly acidic proteins with p*I*'s of 5.85 and 5.60. In bovine tissues, both proteins and a third protein of M_r 35 000 cross-react immunologically with each other and with *Torpedo* calelectrin (M_r 34 000) and are therefore identified as mammalian calelectrins. In all tissues of *Torpedo marmorata* tested, only a single molecular mass form of calelectrin exists, whereas multiple forms of calelectrin exist in mammalian tissues, indicating gene duplication during evolution. We suggest that the evolutionary conservation and diversification, the high tissue concentrations, and the Ca^{2+}-specific interactions of the calelectrins make them candidates for Ca^{2+}-dependent regulators of membrane events in animal cells.

Ca^{2+} acts as a universal cytosolic second messenger by virtue of its interaction with a wide variety of specific proteins (Kretsinger, 1976, 1980). It also couples secretion to excitation in secretory cells and nerve endings (Douglas, 1968), but the effector protein(s) for this process is (are) unknown although the inhibition of secretion by drugs like trifluoperazine and fluphenazine has been taken as evidence for a role for calmodulin (Douglas & Nemeth, 1982; Baker & Knight, 1981; Garofalo et al., 1983). Secretion by exocytosis involves Ca^{2+}-dependent regulation of the cell's membrane traffic which finally results in apposition, fusion, and reendocytosis of the secretory vesicle membranes (Case, 1978; Silverstein et al., 1977). As a model system, the Ca^{2+}-dependent aggregation and fusion of isolated secretory vesicle membranes are frequently used (Morris et al., 1983). Only two proteins have as yet been identified which potentiate this reaction, synexin (Creutz et al., 1978, 1979) and calelectrin (Südhof et al., 1982), while calmodulin, for example, is ineffective (Hong et al., 1982). Calelectrin (M_r 34 000) is a Ca^{2+}-regulated protein isolated from the cholinergic nerve terminals of the electric organ of *Torpedo marmorata* (Walker, 1982) which binds to membranes, self-associates, and promotes membrane aggregation in a Ca^{2+}-dependent and -specific manner. Using immunological methods, we have previously shown that calelectrin also occurs in mammals with a distinctive tissue distribution (Südhof et al., 1983; Walker et al., 1983). While it is present in all classes of human leukocytes, in guinea pig synaptosomes, and in rat and bovine adrenal medulla, it is specifically absent from erythrocytes and adrenal cortical cells. In the adrenal medulla and cholinergic nerve terminals, calelectrin is associated with the secretory vesicles (Walker et al., 1983). We now report the purification and characterization of two calelectrins with differing molecular masses from bovine liver and brain. We also demonstrate that only one molecular mass form exists in *Torpedo marmorata*. We show that the calelectrins have properties and a cellular localization which suggest a role in the Ca^{2+}-triggered membrane traffic.

Experimental Procedures

Preparation of Tissue Fractions. Bovine liver, brain, and adrenal medullas were obtained fresh at a local slaughterhouse and frozen at −80 °C until used. Thawed tissues were minced and homogenized (1 volume of tissue/2 volumes of buffer) in 0.15 M NaCl–10 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES),[1] pH 7.4, containing 2 mM $CaCl_2$ in a Waring blender (2 × 30 s) and in a Potter glass–Teflon homogenizer (two strokes). Ca^{2+}-soluble proteins were separated from Ca^{2+}-insoluble structures by centrifugation for 30 min at 27000g_{max}. The membranes were washed in the same buffer and recentrifuged twice. The third pellet was resuspended in the same buffer containing 5 mM EGTA instead of Ca^{2+}, and the EGTA-solubilized proteins were separated from the membranes by centrifugation at 27000g_{max} for 30 min and at 100000g_{max} for 1 h. In some experiments, the procedures were performed in the presence of 2 mM PMSF, but since there was no difference in the proteins we were studying, it was omitted in further experiments. All steps were performed at 4 °C. Total soluble protein fractions from *Torpedo* and bovine tissues were obtained by homogenizing fresh tissues in 0.4 M NaCl–10 mM Tris–1 mM EGTA–2 mM PMSF for *Torpedo* and in 0.15 M NaCl–10 mM HEPES–5 mM EGTA–2 mM PMSF for bovine tissues, pelleting the membranes by centrifugation (100000g_{max} for 1 h), and retaining the supernatant fluids for chromatography or SDS–PAGE.

Isolation of Calelectrins. (A) Hydrophobic Interaction Chromatography. The tissue EGTA washes were adjusted to 6 mM $CaCl_2$ with 1 M $CaCl_2$ and loaded onto a phenyl-Sepharose column (100-mL bed volume, 3.3 × 30 cm, Pharmacia) preequilibrated with 0.15 M NaCl, 10 mM HEPES, and 0.5 mM $CaCl_2$, pH 7.4. The column was washed with the equilibration buffer until the eluent was protein free

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane; kDa, kilodalton(s).

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and then eluted with 10 mM Tris–10 mM EGTA, pH 7.8. This elution was followed by an elution with 0.1 M Tris, pH 11.0, so that three fractions were obtained (cf. Figure 6). For reuse, the column was extensively washed in 0.1% SDS followed by distilled water. Hydrophobic interaction chromatography of total soluble protein fractions from bovine tissues were performed in the same way except that before the column was eluted with 10 μM Tris–10 μM EGTA it was eluted with 10 mM HEPES–10 μM Tris–0.5 mM Ca^{2+} to desorb nonspecifically bound proteins. This low ionic strength Ca^{2+}-elution step greatly enhanced the specificity of the chromatography scheme when total soluble protein fractions were used (cf. Figure 1).

(*B*) *Chromatofocusing.* Bovine calelectrins found in the EGTA eluent were further purified on a 25-mL chromatofocusing column (0.6 × 30 cm) equilibrated with 25 mM imidazole, pH 6.5, and eluted with Polybuffer diluted 1:8 at pH 4.0 (chromatofocusing columns and Polybuffer were obtained from Pharmacia). In spite of their virtually identical p*I*'s (cf. Figure 4), the two proteins eluted from the chromatofocusing column widely separated from each other.

(*C*) *Gel Filtration.* In some experiments, the proteins were purified from the EGTA eluent by gel filtration on an AcA 44 gel filtration column (1.6 × 145 cm) preequilibrated and eluted with 0.1 M NaCl–1 mM EGTA–0.02% NaN_3 at 17 mL/h. For Stokes radius determinations, the column was calibrated with bovine serum albumin (K_s = 3.55 nm), ovalbumin (K_s = 3.05 nm), chymotrypsinogen A (K_s = 2.09 nm), and ribonuclease A (K_s = 1.64 nm), and the excluded volume was determined by blue dextran and thyroglobulin. The elution volumes of all proteins were checked by SDS–PAGE, and the Stokes radii of the calelectrins were determined by interpolation of the linear plot of $(-\log K_{av})^{1/2}$ vs. the Stokes radius (Laurent & Killiander, 1964). All protein standards were obtained from Pharmacia. All protein concentration steps were performed by precipitation with ammonium sulfate (95% saturation). All purification procedures were carried out at 4 °C except for the gel filtration which was performed at room temperature.

Preparation of Antisera. The antiserum against *Torpedo* calelectrin and the M_r 32 500 protein were raised against the highly purified proteins which were further purified by SDS–polyacrylamide gel electrophoresis. The regions of gels containing the respective proteins were excised from the unstained gels, homogenized (1:5 v/v) in running buffer containing 0.1% SDS, and extracted for 24 h at 20 °C. The polyacrylamide was then removed by filtration, and the filtrates were dialyzed against several changes of water at 4 °C for 48 h. The proteins were then concentrated by freeze-drying. New Zeland white rabbits were immunized on four occasions at weekly intervals with 50 μg of protein mixed with Freund's adjuvant (complete for the first injection and subsequently incomplete). The protein was injected subcutaneously at four sites on the back. The animals were bled 1 week after the final injection. The antiserum against the M_r 67 000 calelectrin was raised against nondenaturated protein. A rabbit was immunized on five occasions at weekly intervals with 80 μg of protein for the first occasion in Freund's complete adjuvant and subsequently 50 μg of protein in Freund's incomplete adjuvant. One week after the final injection, the animal was bled.

SDS–PAGE and Immunoblotting. SDS–PAGE was performed on 11.1% or 15.0% polyacrylamide gels in the presence of mercaptoethanol as described (Walker, 1982). Two-dimensional gels were prepared by the method of O'Farrell (1975). Immunoblots of gels were obtained after electrophoretic transfer of proteins onto nitrocellulose paper as described (Walker et al., 1983). Control stains were made with preimmune or nonspecific rabbit sera and were blank.

Amino Acid Analysis. Amino acid analysis was performed on the purified protein after hydrolysis with 6 M HCl for 6, 18, 24, 48, and 72 h. The results presented were obtained by extrapolation back to zero times. Cysteine was determined independently as described by Spackman et al. (1967). Tryptophan was not determined.

Binding of Calelectrins to Chromaffin Granule Membranes. Chromaffin granule membranes were obtained by lysing and centrifuging pure chromaffin granules prepared in the presence of 1 mM EGTA by the method of Smith & Winkler (1967) as described (Südhof et al., 1982). Chromaffin granule membranes were incubated for 10 min with purified M_r 67 000 or M_r 32 500 protein in Ca^{2+}–EGTA–imidazole buffers at pH 6.8 containing 3.75–4.0 mM EGTA, 75–80 mM imidazole (with a constant EGTA/imidazole ratio of 1/20), and the appropriate Ca^{2+} concentrations to give the required final Ca^{2+}/EGTA ratios. The samples were centrifuged in a Beckman airfuge for 30 min at 28 psi and the protein concentrations in the supernatant fluids determined (all steps at room temperature). Controls with only the purified proteins present showed no pelleting over the Ca^{2+} range measured. Controls with only membranes present demonstrated low, Ca^{2+}-independent protein levels in the supernatants. Binding was calculated as the percent free protein remaining in the supernatant fluid by taking the supernatant's protein concentration with no Ca^{2+} as 100% free protein and the lowest protein concentration (usually at 0.5 mM Ca^{2+}) as 0% free protein.

Potentiation of the Ca^{2+}-Induced Aggregation of Chromaffin Granule Membranes. Chromaffin granule membrane aggregation was measured by tracing the turbidity changes at 540 nm in a PMQ 3 Zeiss spectrophotometer as a function of time and Ca^{2+} concentration (Südhof et al., 1982). The chromaffin granule membrane concentration was adjusted to give an OD_{540} of ca. 0.040. Assays were performed in 0.080 M imidazole and 40 mM KCl at pH 6.8 with 0.1 mM EGTA and started by the addition of Ca^{2+}. Control proteins [bovine serum albumin, parvalbumin, poly(aspartate), poly(glutamate), and poly(alanine)] were obtained from Sigma (Munich, FRG) and used without further purification.

Ca^{2+}-Binding Measurements. Experiments to demonstrate the Ca^{2+}-binding property of the proteins isolated were performed by gel filtration (Hummel & Dreyer, 1962). The purified proteins were extensively dialyzed against 0.1 M Tris, 0.1 M NaCl, and 0.02% NaN_3, pH 8.0, and then diluted 1:1 with water containing $^{45}Ca^{2+}$ (obtained from New England Nuclear, Dreieich, FRG) to give final concentrations of 1 and 10 μM Ca^{2+}. After 30-min incubation, the sample was chromatographed over a Sephadex G-25 column (0.9 × 15 cm) preequilibrated with the same buffer, and 1-min fractions (1 mL) were collected and counted. Concentration peaks in the excluded volume followed by troughs were observed which are typical for binding. In connection with this experiment, only plastic containers were used, and all solutions were made in Millipore demineralized water.

Calculation of Free Ca^{2+} Concentrations. This was performed with the method of Caldwell (1970) using a pK_d of 6.29 for Ca^{2+}–EGTA at pH 6.8 (Sillen et al., 1964).

Protein Concentrations. These were determined according to Bradford (1976).

Results

Isolation of Mammalian Calelectrins. When bovine liver,

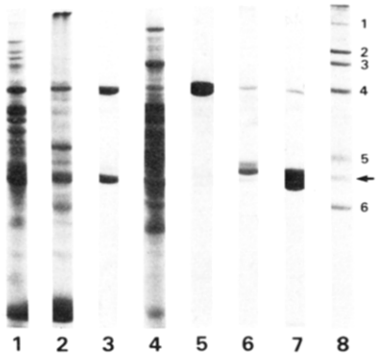

FIGURE 1: Coomassie Blue stained SDS–polyacrylamide gels (11.1%) of the different purification steps of mammalian calelectrins from liver. Lane 1, EGTA wash; lane 2, proteins from the EGTA was not binding to the phenyl-Sepharose column in the presence of 0.15 M NaCl, 10 mM HEPES, and 0.5 mM Ca^{2+}; lane 3, proteins eluted from the phenyl-Sepharose column with 10 mM Tris–10 mM EGTA; lane 4, proteins eluting with 0.1 M Tris; lane 5, purified M_r 67 000 protein from chromatofocusing column; lane 6, intermediate peak composition from chromatofocusing column; lane 7, purified M_r 32 500 protein from chromatofocusing column; lane 8, molecular weight standards: (1) myosin (M_r 205 000), (2) β-galactosidase (M_r 116 000), (3) phosphorylase *b* (M_r 97 400), (4) bovine serum albumin (M_r 67 000), (5) ovalbumin (M_r 45 000), (6) carbonic anhydrase (M_r 29 000); the arrow indicates *Torpedo* calelectrin. Purified proteins are purposely overloaded to show minor contaminants.

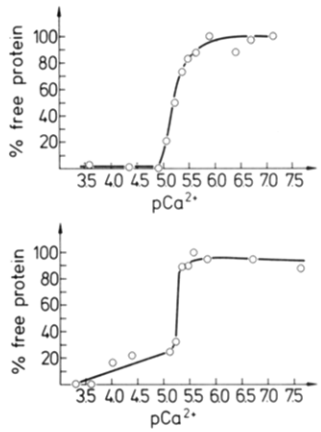

FIGURE 2: Ca^{2+}-dependent binding of purified M_r 67 000 (upper graph) and M_r 32 500 (lower graph) calelectrins to chromaffin granule membranes. The graph depicts the percent free (unbound) protein as a function of free Ca^{2+} concentration as determined by a centrifugation assay (cf. Experimental Procedures), clearly demonstrating a very narrow Ca^{2+} concentration range over which binding to membranes is regulated with half-maximal binding at ca. 5.5 μM Ca^{2+}.

brain, or adrenal medullas are homogenized in 0.15 M NaCl, 10 mM HEPES, pH 7.4, and 2 mM $CaCl_2$ and washed extensively in this buffer by centrifugation and resuspension, the subsequent treatment of the membranes with the same buffer containing 5 mM EGTA leads to the solubilization of several proteins, the most prominent of which are proteins with molecular weights of 32 500 and 67 000 on SDS–PAGE. These proteins are, like calmodulin, specifically retained on hydrophobic phenyl-Sepharose columns in the presence of 0.5 mM $CaCl_2$ and eluted with EGTA (Figure 1). The EGTA column eluent from EGTA washes of membranes contains only two proteins as major constituents (Figure 1) of M_r 67 000 and 32 500. These proteins can be purified on a chromatofocusing column almost to homogeneity (M_r 67 000 protein ≫99% pure; M_r 32 500 protein >95% pure as estimated from densitometric scans of Coomassie Blue stained gels) as shown in Figure 1 for a liver preparation with heavily overloaded gels. With brain and adrenal medullas, similar results are obtained, except that a third protein of M_r 35 000 becomes more prominent (cf. Figure 6). For a typical preparation, 2–3 mg of each protein is obtained per 100 g of tissue (wet weight). The binding of the proteins to phenyl-Sepharose is Ca^{2+} specific with respect to Mg^{2+}, and the isolated proteins can be rebound to and reeluted from fresh columns. The proteins can also be purified by Ca^{2+}-dependent hydrophobic interaction chromatography of the total soluble tissue proteins. Again, the EGTA eluents of phenyl-Sepharose columns contain the proteins of M_r 67 000 and 32 500 as major components at concentrations even higher than calmodulin (Figure 6).

Ca^{2+}-Dependent Interactions. The binding of the purified M_r 67 000 and 32 500 proteins to isolated chromaffin granule membranes was assayed as a function of free Ca^{2+} concentrations in Ca–EGTA buffers by a centrifugation assay. Chromaffin granule membranes were chosen as model membranes because they can be obtained in high yield and purity and are well characterized. As shown in Figure 2, the binding of both proteins to chromaffin granule membranes is regulated over a very narrow Ca^{2+} concentration range with half-maximal binding at 5.5 μM free Ca^{2+}. The change in the Ca^{2+} concentration from 0% to 100% binding was so narrow that in most experiments it was very difficult to obtain intermediate points. However, in the presence of Mg^{2+} at concentrations up to 10 mM, no binding occurred.

The question arises whether the proteins we isolated bind Ca^{2+} in solution or are dependent on membranes for their Ca^{2+} interactions. We measured the Ca^{2+}-binding ability of the calelectrins by the Hummel–Dreyer gel filtration method using radioactive Ca^{2+} at total concentrations of 1 and 10 μM in 50 mM NaCl, 50 mM Tris, and 0.02% NaN_3 at pH 8.0. At both Ca^{2+} concentrations, both the M_r 67 000 and M_r 32 500 calelectrins gave Ca^{2+} peaks in the excluded volume followed by troughs which are characteristic of Ca^{2+} binding (data not shown). Therefore, both proteins bind Ca^{2+} at physiological concentrations.

The purified proteins were also tested for their ability to potentiate the Ca^{2+}-triggered aggregation of chromaffin granule membranes in a turbidity assay ("synexin-like activity"). Figure 3 demonstrates that both proteins were highly active. The kinetics of the calelectrin-potentiated aggregation can be seen in Figure 3A for the M_r 32 500 calelectrin, showing as observed for *Torpedo* calelectrin a very fast phase and a second slow phase (Südhof et al., 1982). A plot of the aggregation amplitudes after 6 min as a function of free Ca^{2+} concentration is shown in panels B and C of Figure 3 for the M_r 67 000 and M_r 32 500 calelectrins, respectively, to demonstrate the Ca^{2+} dependency of the activity. Both proteins seem to have similar Ca^{2+} dependencies in activating the membrane aggregation, but the M_r 32 500 calelectrin apparently is a much more potent aggregator than the M_r 67 000 calelectrin. Clearly, no aggregation occurs below Ca^{2+} concentrations of 10 μM. With Mg^{2+} at any concentration, no aggregation enhancement was ever seen, confirming the Ca^{2+} specificity of the proteins studied. Controls with the proteins or the membranes alone had maximal amplitudes of less than 5% of the combination of both and are therefore omitted. Further controls to test the specificity of the effect of the proteins on Ca^{2+}-induced membrane aggregation were performed in the presence of the following proteins: bovine serum albumin, parvalbumin, poly(glutamate), poly(aspartate), and poly(alanine). None of these had any effect on the Ca^{2+}-induced aggregation of chromaffin granule membranes.

Biochemical and Immunochemical Properties. The purified M_r 67 000 and 32 500 calelectrins eluted well separated in the included volume of an AcA 44 gel filtration column with

Table I: Amino Acid Composition of the Isolated Proteins

amino acid [b]	67-kDa protein		32.5-kDa protein		*Torpedo* calelectrin [a]	
	molar ratio [c]	mol %	molar ratio [c]	mol %	molar ratio [c]	mol %
aspartic acid/asparagine	56	10.5	32	11.4	35	12.8
threonine	28	5.3	16	5.7	17	6.2
serine	44	8.3	18	6.4	19	6.9
glutamic acid/glutamine	70	13.2	38	13.6	37	13.5
proline	14	2.6	5	1.8	8	2.9
glycine	48	9.0	22	7.9	20	7.3
alanine	44	8.3	24	8.6	24	8.8
valine	14	2.6	11	3.9	15	5.5
cysteine	6	1.1	4	1.4	4	1.5
methionine	14	2.6	6	2.1	4	1.5
isoleucine	26	4.9	16	5.7	15	5.5
leucine	52	9.8	30	10.7	25	9.1
tyrosine	14	2.6	9	3.2	8	2.9
phenylalanine	16	3.0	10	3.6	8	2.9
lysine	48	9.0	20	7.1	18	6.6
histidine	8	1.5	4	1.4	5	1.8
arginine	30	5.6	15	5.4	12	4.4
	68.4 [d]		36.3 [d]		35.9 [d]	

[a] From Walker (1982). [b] Tryptophan was not determined. [c] Based on the estimated molecular weight. [d] Molecular weight calculated as the nearest fit of the amino acid composition to the molecular weight determined by SDS-PAGE.

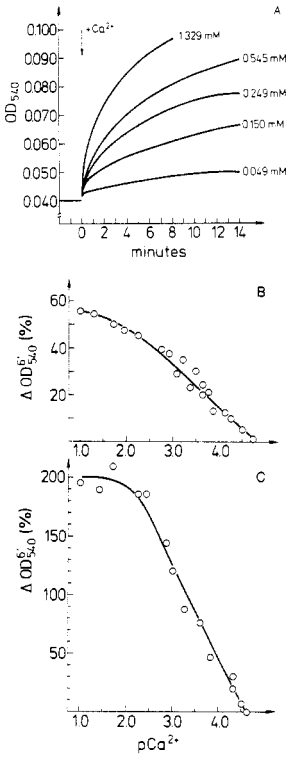

FIGURE 3: Potentiation of the Ca^{2+}-induced aggregation of chromaffin granule membranes by the calelectrins. In panel A, the kinetics of the potentiation of membrane aggregation by the M_r 32 500 calelectrin are shown (numbers give final Ca^{2+} concentrations). Panels B and C are plots of the relative amplitudes of the turbidity changes (in percent of starting OD) after 6 min as a function of the Ca^{2+} concentration for the M_r 67 000 and 32 500 calelectrins, respectively. Assays were performed in 80 mM imidazole, 40 mM KCl, and 0.1 mM EGTA, pH 6.8, with 16 μg/mL M_r 67 000 and 20 μg/mL M_r 32 500 calelectrins and chromaffin granule membrane concentrations to give starting OD's of 0.040. The graphs clearly demonstrate that there is no significant aggregation enhancement at Ca^{2+} concentrations below 10 μM. Furthermore, the M_r 32 500 calelectrin seems to be a much more potent aggregator than the M_r 67 000 calelectrin.

apparent Stokes radii of K_s = 3.40 and 2.53 nm, respectively (data not shown). These Stokes radii correspond to approximate molecular weights of 66 000 and 32 000, demonstrating that the proteins occur as monomers in solution. The amino acid compositions of the purified proteins have been analyzed after HCl hydrolysis and are given in Table I together with the amino acid composition of *Torpedo* calelectrin (Walker, 1982). All three proteins have extremely similar compositions. The molecular weights calculated from the amino acid compositions are 68 400 for the M_r 67 000 protein and 36 300 for the M_r 32 500 protein. When tested for heat stability, both proteins were found to be very heat sensitive, precipitating at a temperature as low as 70 °C.

In order to test for immunological cross-reactivity, an antibody was prepared against gel-purified denatured *Torpedo* calelectrin. Two-dimensional gels (first dimension, isoelectric focusing; second dimension, SDS–PAGE) were run of EGTA eluents from the Ca^{2+}-dependent hydrophobic interaction chromatography columns (Figure 4a), of the total soluble protein fraction from *Torpedo* electric organ (Figure 4c), and of a mixture of both (Figure 4e). After being stained for protein and destained, the gels were immunoblotted electrophoretically with the antiserum to *Torpedo* calelectrin. Figure 4 shows that while in the total soluble protein fraction from the *Torpedo* electric organ only one protein, calelectrin, is stained with the antiserum (Figure 4d), both of the proteins we isolated cross-react with the antiserum (Figure 4f). The two-dimensional gels also allowed accurate determination of the p*I*'s of the proteins both from the directly measured pH gradient in the gel (Figure 4b) and by reference to internal standards of defined p*I* [actin, 5.5; M_r 43 000 protein, 6.1 (Witzemann et al., 1983)] which are present in the soluble fraction from *Torpedo* electric organ. The p*I*'s determined are very similar for both proteins, namely, 5.85 for the M_r 67 000 protein and 5.60 for the M_r 32 500 protein. It seemed puzzling that there should be high and low molecular weight calelectrins in bovine tissues with the high molecular weight form twice the mass of the low molecular weight form but not in *Torpedo marmorata*. To substantiate this finding, we immunoblotted total soluble protein fractions from various *Torpedo* tissue (Figure 5). Clearly, only a single molecular weight form (M_r 34 000) of calelectrin is observed. Since each lane contains identical amounts of protein, this experiment also allows a semiquantitative estimation of the tissue distribution of calelectrin in *Torpedo*. The highest concentrations are found in the electric organ, electromotor nerve, and electric lobe. Secretory tissues like liver, kidney, intestine, and pancreas contain intermediate concentrations of calelectrin, while

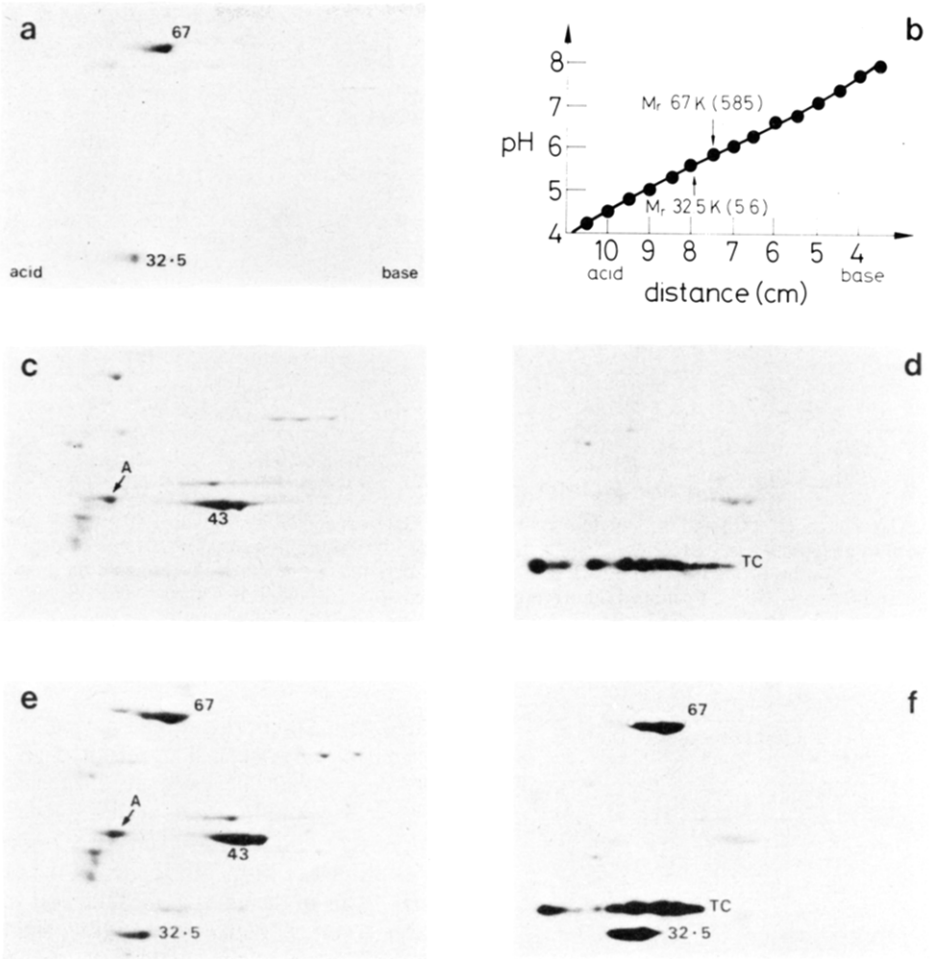

FIGURE 4: Two-dimensional gel electrophoresis and immunoblots of bovine and *Torpedo* calelectrins: (a) Coomassie Blue stained 2D gel of the hydrophobic interaction chromatography EGTA eluent; (b) pH profile of the isoelectric focusing gel; (c) Coomassie Blue stained 2D gel of the total soluble proteins from *Torpedo* electric organ; (d) electrophoretic immunoblot of (c) with *Torpedo* calelectrin antiserum after extensive destaining; (e) Coomassie Blue stained mixture of (a) and (c); (f) immunoblot of (e). The immunoblots show that the *Torpedo* calelectrin antiserum is specific for *Torpedo* calelectrin (TC) in the total soluble protein from *Torpedo* electric organ but cross-reacts with both the M_r 67 000 and the M_r 32 500 proteins. In the Coomassie-stained gels, actin (A, p*I* 5.5) and the M_r 43 000 protein of the electric organ (p*I* 6.1) are indicated and provide internal p*I* standards.

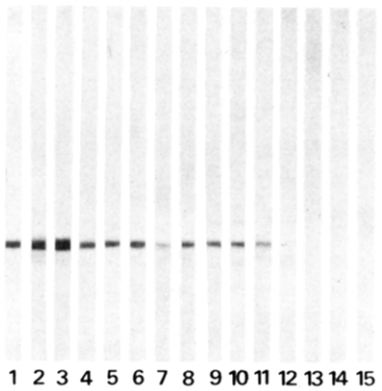

FIGURE 5: Immunoblots of the total soluble proteins from various tissues from *Torpedo marmorata* stained with *Torpedo* calelectrin antiserum. Each lane was loaded with 50 μg of protein from the following tissues: (1) electric lobe; (2) electromotor nerve; (3) electric organ; (4) brain; (5) spinal cord; (6) liver; (7) pancreas; (8) kidney; (9) intestine; (10) uterus; (11) heart; (12) speen; (13) blood; (14) back muscle; (15) gills.

no significant amounts can be detected in tissues like gills, spleen, and back muscle.

In contrast to *Torpedo* tissues, bovine tissues invariably contained three proteins cross-reacting with *Torpedo* calelectrin, the M_r 67 000 and 32 500 proteins we isolated and a third protein of M_r 35 000 present in low concentrations in liver EGTA eluents but in much higher concentrations in adrenal medullas and brain EGTA eluents (Figure 6). To study the immunologic cross-reactivities among these proteins, we have raised antibodies against native M_r 67 000 calelectrin and against gel-purified, denatured M_r 32 500 calelectrin (both from liver). As demonstrated in Figure 6, the *Torpedo* calelectrin antiserum stains the mammalian low molecular weight calelectrins slightly more strongly than the M_r 67 000 calelectrin, while the antibody against denatured M_r 32 500 calelectrin intensely stains all three calelectrins. In contrast, the antiserum against native M_r 67 000 calelectrin have very little immunological cross-reactivity toward the M_r 32 500 calelectrin. This cross-reactivity pattern is further evidence that there is no monomer–dimer relationship between low and high molecular weight calelectrins and that they constitute a family of related but distinct proteins.

Discussion

We have reported here the identification and purification of two new Ca^{2+}-binding proteins of M_r 67 000 and 32 500 from bovine liver, brain, and adrenal medulla. These proteins were characterized biochemically as summarized in Table II. The biochemical properties of the isolated proteins are very similar to those for *Torpedo* calelectrin, and our immunochemical studies demonstrate that the two proteins we isolated

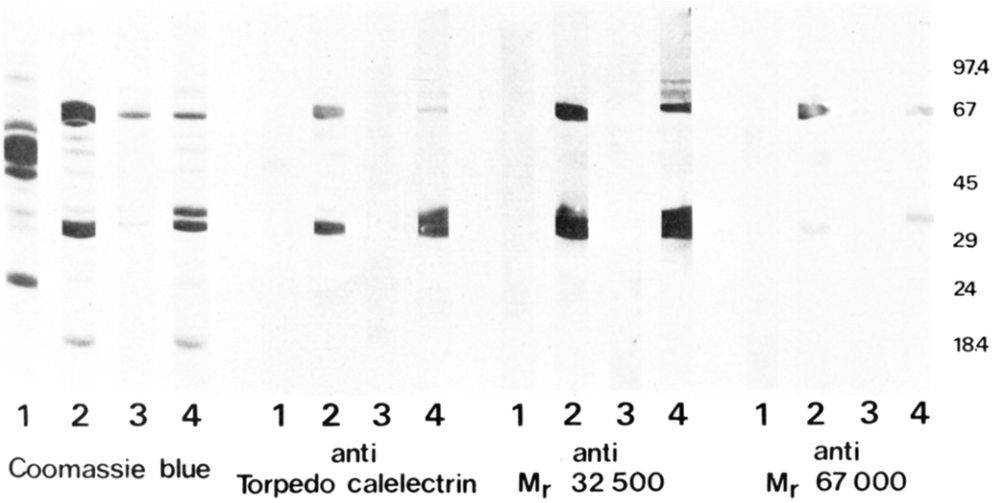

FIGURE 6: SDS–polyacrylamide gel (15.0%) and immunoblot analysis of the Ca^{2+}-dependent hydrophobic interaction chromatography fractions from columns loaded with total soluble tissue proteins. Total soluble proteins ($100000g_{max}$ supernatant) from bovine liver (lanes 1 and 2) and adrenal medulla (lanes 3 and 4) were brought to 0.5 mM free Ca^{2+} and loaded onto a phenyl-Sepharose column preequilibrated with 0.5 M NaCl, 10 mM HEPES, and 0.5 mM Ca^{2+}. Proteins bound were first eluted with 10 mM Tris, 10 mM HEPES, and 0.5 mM Ca^{2+}, pH 8.0 (lanes 1 and 3) and then with 10 mM Tris and 10 mM EGTA, pH 8.0 (lanes 2 and 4). Blots of all four fractions were run with *Torpedo* calelectrin antiserum, bovine M_r 32 500 antiserum, and bovine M_r 67 000 antiserum, demonstrating that the main components selectively eluting in the presence of EGTA, proteins of M_r 67 000 and 32 500 in liver and of M_r 67 000, 35 000, and 32 500 in adrenal medullas, are calelectrins.

Table II: Summary of the Biochemical Properties of the Purified Proteins

property	method	values
molecular weight	SDS-PAGE	32 500, 67 000
molecular weight	amino acid composition	36 300, 68 400
Stokes radius (nm)	gel filtration	2.53, 3.40
isoelectric point	isoelectric focusing	5.60, 5.85
Ca^{2+} binding at 1 and 10 μM Ca^{2+}	gel filtration	yes, yes
half-maximal binding to membranes at [Ca^{2+}] (μM)	centrifugation	5.5, 5.5
potentiation of membrane aggregation at [Ca^{2+}] (μM)	turbidity	>10, >10

and a third protein of M_r 35 000 selectively cross-react with *Torpedo* calelectrin, while in *Torpedo* tissues only a single molecular weight form of calelectrin (M_r 34 000) exists. We therefore identify the isolated Ca^{2+}-binding proteins as mammalian calelectrins. The presence of multiple forms of calelectrin with limited cross-reactivities in bovine tissues as compared to the presence of only a single form in *Torpedo marmorata* suggests the possibility of gene duplication during evolution.

The calelectrins can be purified by Ca^{2+}-dependent hydrophobic interaction chromatography like calmodulin, and we are therefore able to identify three of the previously observed proteins which copurify with calmodulin on hydrophobic affinity columns as calelectrins (Moore & Dedman, 1982). Furthermore, the proteins previously observed by Pollard & Scott (1982) as the main components of the Ca^{2+}-insoluble but EGTA-soluble total liver membrane fractions may be identical with the calelectrins. However, identity of the calelectrins with the proteins listed below can be ruled out. Both the M_r 32 500 and the M_r 67 000 proteins do not have any protein kinase activity, in the presence of either Ca^{2+} with phospholipids or calmodulin (T. C. Sühof, unpublished results), and are therefore not related to the protein kinase described by Schatzman et al. (1983). The calelectrins have amino acid compositions distinctly different from fimbrin, a Ca^{2+}-regulated M_r 68 000 protein, and its solubilization procedure is exactly the opposite, making identity highly unlikely (Bretscher & Weber, 1980; Glenney et al., 1981). The equal distribution of calelectrin in liver and brain excludes any relation to the M_r 68 000 neurofilament subunit (Osborn & Weber, 1982). The distinctive tissue distribution of the calelectrins, including high levels in the adrenal medulla and very low levels in the adrenal cortex (Walker et al., 1983), and their heat sensitivity make identity of the calelectrins and clathrin light chains improbable (Mello et al., 1980). In summary, we think that identity of the calelectrins with any previously characterized, well-established protein is unlikely. Our results suggest that the calelectrins are Ca^{2+}-binding and Ca^{2+}-regulated proteins with an unknown function related to Ca^{2+} as a second messenger. While there can be little doubt about the physiological significance of the Ca^{2+} binding by the calelectrins and their Ca^{2+}-dependent membrane binding [its Ca^{2+} requirements equal those of exocytosis in model systems (Wilson & Kirshner, 1983)], the Ca^{2+} requirements for membrane aggregation are rather high. On the other hand, controls have shown that this activity is not an incidental property of several proteins but, to our knowledge, restricted to calelectrins and to synexin, the protei n for which it was first described and which has no lower Ca^{2+} requirements (Creutz et al., 1978, 1979). The differences in the Ca^{2+} requirements for membrane binding and membrane aggregation could be explained by two models: either calelectrin contains two membrane-binding sites with differing Ca^{2+} affinities or membrane aggregation occurs via protein–protein bridges, the formation of which has a higher Ca^{2+} requirement than the membrane binding. In summary, although the molecular processes governing membrane traffic and fusion during exo- and endocytosis are unknown (Baker & Knight, 1981), the properties of the calelectrins, like evolutionary conservation, Ca^{2+}-specific membrane interactions, and enrichment at sites of exocytosis, point to a role in these processes.

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Kinetics of Cytochrome P-450 Reduction: Studies in Bovine Adrenocortical Microsomes†

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ABSTRACT: The results of the present study indicate first that in the microsomal preparation, the components of the P-450 reduction system are heterogeneously distributed, comprising dissociable and nondissociable parts. Second, the P-450 reduction curve can be adequately described by a sum of two exponential functions, indicating two concurrent first-order reactions. Third, the two phases can be altered independently. The addition of the substrate increased the extent of the fast phase while it had little or no effect on that of the slow phase. Changes in the interaction of the dissociable and nondissociable components affected the extent of the slow phase while they were without effect on that of the fast phase. Experiments with different steroids indicated that the independence of the two phases is not due to functionally different P-450's and that the cytochrome reduced in both phases is essentially P-450$_{C\text{-}21}$. The results are interpreted as follows: Transformation of P-450 from the low- to the high-spin state controls the total P-450 reduced. The rate and the biphasicity of the reduction are functions of the interaction of P-450 and the reductase.

Previous investigations (Narasimhulu et al., 1966, 1971a,b) indicated that the substrate-produced type I spectral change in cytochrome P-450 represents transformation of the cytochrome from an "inactive" to an "active" state and that this transformation is essential for the enzymatic reduction of the cytochrome. It is now generally agreed that the spectral change is associated with the transformation of the heme from a low-spin to a high-spin state and that this is an obligatory step for the transfer of the first electron to the cytochrome (White & Coon, 1980). The process of the first electron transfer has been investigated under anaerobic conditions in various laboratories. Gigon et al. (1969) first noted that in

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