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PREPARATION AND CHARACTERIZATION OF CALCIUM-BINDING AND OTHER HYDROPHOBIC PROTEINS FROM SYNAPTIC MEMBRANES

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SUMMARY

A number of hydrophobic proteins have been separated and purified to varying degrees from synaptic membranes derived from bovine brain. The proteins, which have been obtained using preparative acrylamide gel electrophoresis, have been analyzed for molecular weight, amino acid composition, peptide mapping, N-terminal amino acids, and for their ability to bind calcium and ATP. A number of the proteins bound calcium, the greatest binding being associated with a component having a molecular weight of $1.6 \cdot 10^4$, a binding capacity of 4 calcium/molecule, and a K_m of $1.5 \cdot 10^{-5}$ M. An acidic tryptic peptide derived from this protein was evidently responsible for the calcium-binding. ATP binding appeared to be confined largely to the higher molecular weight proteins. From the peptide mapping there appears to be a similar acidic component in a number of the proteins exhibiting calcium-binding. ATP-binding was associated mainly with the high molecular weight proteins, particularly those which consisted of numerous basic tryptic peptides.

INTRODUCTION

In an effort to elucidate the role of Ca^{2+} in cellular function several types of Ca^{2+}-binding proteins have been isolated from various animal tissues. Included among these are troponin [1]; an intestinal protein thought to be involved in the adsorption of dietary Ca^{2+} [2]; several proteins from sarcoplasmic reticulum believed to be responsible for sequestering Ca^{2+} [3]; an acidic phosphoprotein from porcine brain and bovine adrenal medulla, presumably specific for neuronal tissue [4]; and an axoplasmic protein from the giant squid axon [5]. With one exception, all of the above-mentioned proteins are water-soluble and are, therefore, readily purified by conventional procedures. The one exception is the protein from sarcoplasmic reticulum, which is initially solubilized in detergent but is subsequently soluble in aqueous media after purification.

Interest in this laboratory has focused on the characteristics and possible function of hydrophobic proteins derived from isolated synaptic membranes of mammalian brains [6, 7]. Attempts to purify and separate such proteins after solubili-

zation in dodecyl sulfate and other detergents by the use of gel filtration and column chromatography have not been very successful. A high molecular weight complex of such proteins has been shown to exhibit both Ca^{2+} and ATP binding as well as $(Na^+ + K^+)$-ATPase and 5'-nucleotidase activity. From an analysis of N-terminal amino acids and acrylamide gel electrophoresis with dodecyl sulfate, it could be shown that the material was quite heterogeneous, even after repeated runs on gel filtration columns.

In the present study a number of the components of the hydrophobic complex have been separated and purified by the use of preparative gel electrophoreses in dodecyl sulfate. The individual components have been analyzed for N-terminal amino acid, amino acid composition, and peptide profile, as well as for their ability to bind Ca^{2+} and ATP. The major effort has been devoted to the purification and characterization of a Ca^{2+}-binding protein having a molecular weight of about 16 000.

MATERIALS AND METHODS

Preparation of synaptic membranes from calf brain

Approx. 00 g of cerebral gray matter from the brains of freshly killed calves were homogenized thoroughly in large glass homogenizers (capacity 50 ml; clearance 0.2 mm) in 15 times its volume of 0.32 M sucrose. After removal of the heavier components by centrifugation twice at $1000 \times g$ for 10 min, a lighter pellet was obtained by centrifugation at $15\,000 \times g$ for 20 min. This pellet was then homogenized in 60 ml of 0.32 M sucrose and 8 ml of suspension layered on cellulose centrifuge tubes containing 14 ml layers each of 1.2 M and 0.8 M sucrose. After centrifugation at $100\,000 \times g$ for 1 h in a Spinco 65 L ultracentrifuge using a SW 27 rotor, the intermediate layer containing nerve endings was removed by gentle suction and retained. This layer was then suspended in 200 ml of H_2O, homogenized, kept at 0 °C for 1 h, and then centrifuged at $100\,000 \times g$ for 30 min to yield a pellet of disrupted nerve endings.

The pellet containing disrupted nerve endings was then rehomogenized in 84 ml of H_2O and 7 ml layered on gradient tubes containing 6 ml each of the following sucrose solutions in a discontinuous gradient: 1.2 M, 1.0 M, 0.8 M, 0.6 M, 0.4 M. After centrifugation at $100\,000 \times g$ for 1 h, the various layers were removed by gentle suction and designated with the letters, D–I, beginning from the top. The layers were washed twice by being homogenized in 20 vol. of H_2O and centrifuged at 50 000 $\times g$ for 20 min.

Preparative gel electrophoresis of hydrophobic proteins

The method used was similar to that of Neville [8] for analytical gel electrophoresis. The preparative gels were prepared in glass columns 12 cm long and 1.8 cm diameter. Each tube contained 30 ml of separation gel comprised of 11 % polyacrylamide and 3 ml of stacking gel containing 3 % polyacrylamide. To each gel an 0.4 ml sample was applied containing the equivalent of 5 mg protein from fraction G, dissolved in the upper buffer+2 % dodecyl sulfate+2 % mercaptoethanol. The cylindrical electrophoresis chamber, which processed a total of 8 gels, was constructed of plexiglass and consisted of a lower and upper reservoir, having volumes of 300 and 400 ml respectively. The upper buffer consisted of 0.04 M boric acid/0.041 M Tris/

0.1 % dodecyl sulfate, pH 8.64; and the lower buffer, 0.42 M Tris · HCl, pH 9.18. Gels were prepared as described by Neville [8]. In all procedures the sodium salt of dodecyl sulfate was used.

Electrophoresis was performed at 24 °C for a period of 8 h at a current of 5 mA/tube for the first h and 10 mA/tube for the remaining time. Coomassie blue was used as a marker to determine completion of the run. After electrophoresis, a narrow (0.2 cm thick) longitudinal section of each gel was stained with 0.25 % Coomassie blue in 50 % methanol/7 % acetic acid. Upon removal of the excess dye with 7 % acetic acid the stained strip was matched with the unstained portion which was then sectioned transversely into 9 parts, numbered I–IX beginning at the origin. Each section consisted of a major band although minor components were also present in some of the sections (see Results).

Extraction of proteins from the gel

In order to separate the proteins from the gel it was necessary to use dodecyl sulfate in combination with electrophoresis. After the various segments were minced and suspended in 0.2 % dodecyl sulfate, pH 8.0, they were transferred to a 125 ml bottomless plastic bottle with cellophane dialysis tubing tied to its neck. A fine nylon membrane (37 μm mesh), attached to the neck by means of a plastic screw cap, prevented the gel fragments from entering the dialysis bag. The dialysis bag was then immersed into a lower chamber containing 0.2 % dodecyl sulfate, pH 8.0. Electrophoresis was performed at 24 °C with a current of 30–40 mA for 4 h, with the anode (Pt wire) inserted into the lower chamber.

After the contents of the dialysis bag were centrifuged at 40 000 $\times g$ for 30 min, the supernatant was lyophilized. The dried residue was then washed 3 times with 10 ml volumes of methanol and twice with 10 ml volumes of 50 % methanol. With the use of dodecyl ^{35}S-sulfate (Amersham/Searle Corp.; spec. act., 10 mCi/mmol) it was determined that the washing procedure removed over 99.90 % of the dodecyl sulfate in the residue. The washing with 50 % methanol was necessary to remove most of the acrylamide associated with the residue. The protein residues were then suspended in a small volume of H_2O and stored at −20 °C.

Preparation of larger amounts of protein IX

In order to prepare larger quantities of protein IX, advantage was taken of the fact that it was the fastest moving component on the gel. Gel electrophoresis was performed as described above except that the equivalent of 10 mg of protein from fraction G was applied to each gel and a dialysis bag, filled with 25 ml of and immersed in the lower buffer, was attached to each gel. The length of the gel was reduced to 8 cm. Since the mobility of the protein relative to the dye indicator was known, the electrophoresis time for elution of the protein IX could be accurately determined. The protein was recovered by first centrifuging the contents at 100 000 $\times g$ for 30 min, dialyzing the supernatant against distilled H_2O at 4 °C followed by lyophilization, and then washing the residue with 50 % methanol to remove the remaining dodecyl sulfate. The protein was dried in vacuo and stored at −20 °C.

Analytical gel electrophoresis

All 9 proteins were submitted to analytical acrylamide gel electrophoresis,

under conditions identical to the preparative procedure, and exhibited patterns and mobilities very similar to those shown for the individual components in Fig. 3 (data not shown).

Peptide mapping

Peptide mapping was performed by the procedure of Furlan and Beck [9] with some modifications. Approximately 1 mg of each of the proteins was homogenized in 0.05 % dodecyl sulfate in 0.2 M ammonium carbonate, pH 8.5 and incubated with 20 μg trypsin for 4 h at 37 °C. The trypsin had been treated with diphenylcarbamyl chloride to inactivate chymotrypsin. After the trypsin-digest was lyophilized, it was redissolved in 50 μl of distilled H_2O and 10 μl aliquots applied to thin-layer chromatography plates (20×20 cm) coated with a 0.5 mm layer of powdered MN 300 cellulose. Electrophoresis was then carried out in a mixture of pyridine/acetic acid/water (20 : 7 : 973) at pH 5.5 for 60 min at 1000 V. After the plates were dried for 1 h at 50 °C, they were subjected to ascending chromatography in a solution containing *n*-butanol/acetic acid/H_2O (4 : 1 : 5). After the plates were dried at 100 °C, they were sprayed with 0.05 % fluorescamine in acetone and visualized under ultraviolet light.

Amino acid analysis

Hydrolysis of the protein samples was carried out in sealed ampoules under N_2 for 48 h at 110 °C. Amino acid analyses were performed on the Beckman 120 amino acid analyzer. Methionine was determined as methionine sulfone, and half-cystine as cysteic acid after the samples had been oxidized by performic acid, prior to acid hydrolysis.

Acidic peptide from the Ca^{2+}-binding protein

After 8 mg of protein IX had been subjected to trypsin digestion, the digest was applied equally to 4 thin-layer chromatography plates, and separated as described in the section Peptide mapping. A peptide near the origin of the thin-layer chromatography plate (marked Pt_9 in Fig. 4f) was scraped off with a razor blade and extracted from the cellulose with 6 M HCl at 50 °C for 15 min. The dodecyl sulfate extract was assayed for Ca^{2+}-binding; while the HCl-extract was hydrolyzed for 24 h at 100 °C and amino acid analysis performed on the amino acid analyzer. In order to study Ca^{2+}-binding the peptide was extracted from the cellulose with 2 ml of 2 % dodecyl sulfate. After centrifugation the extract was lyophilized and the residue washed 3 times with methanol to remove the dodecyl sulfate. It was suspended in 10^{-5} M dodecyl sulfate, homogenized, and assayed for Ca^{2+}-binding.

End group analysis

N-terminal amino acid analysis was performed according to the method of Gray [10], except for the addition of 0.1 % dodecyl sulfate, needed to dissolve the protein. Prior to dansylation the proteins were extracted twice with chloroform/methanol (2 : 1) in order to remove all lipids. After dansylation at 27 °C for 4 h, the proteins were precipitated with 5 % trichloroacetic acid, washed twice with 50 % methanol to remove the acid and dodecyl sulfate, and then hydrolyzed with 6 M HCl in an N_2 atmosphere for 18 h. Two-dimensional cellulose thin-layer chromatography was used to identify the dansylated N-terminal amino acids according to the method of Deyl and Rosmus [11].

Measurement of Ca^{2+}-binding to proteins by ultrafiltration

The measurement of Ca^{2+}-binding was performed by the ultrafiltration method of Paulus [12], using an ultrafiltration cell obtained from Metalloglass, Inc. An 0.5 ml incubation medium contained 50 μg protein, 1 mM Tris · HCl buffer, pH 7.5, 1 μCi $^{45}CaCl_2$ (spec. act., 10 Ci/g), $5 \cdot 10^{-6}$ M $CaCl_2$. After 20 min incubation at 27 °C, 0.2 ml was transferred to a UM-10 (Aminco Corp.) filter inserted in the ultrafiltration cell. After the aliquot was filtered within 10 min at a pressure of 40 lb/inch^2, the bottoms of the filters were washed with 5 ml of ethyleneglycol. The filters were then removed and transferred to scintillation vials. Bray's solution was then added and radioactivity was measured in a liquid scintillation spectrometer.

Measure of ATP and Ca^{2+}-binding by a surface film technique

An alternative method for measuring binding of a radiolabeled ligand to protein is the surface film technique [7]. Briefly, it consisted of determining the adsorption of $^{45}Ca^{2+}$ or [^{14}C]ATP from the subsolution to a protein film spread at the surface. Measurements were carried out in teflon planchets (2.0 cm diameter, 0.5 cm deep) containing a final volume of 1.2 ml test solution, and radioactivity was determined by means of a scaler and Geiger-Muller tube. The test solution contained either 0.01 μCi [^{14}C]ATP (spec. act., 5 Ci/mol) in 10^{-5} M ATP or 0.01 Ci $^{45}CaCl_2$ (spec. act., 10 Ci/g) in 10^{-5} $CaCl_2$. The pH for Ca^{2+}-adsorption was 7.5 and for ATP-adsorption, 4.5, adjusted with NaOH or HCl. 5 μl of a protein solution was applied to the surface, prepared by homogenizing the protein in 10^{-6} M dodecyl sulfate, pH 7.5, so that the final concentration was about 2 mg/ml. It could be demonstrated that a solution of 10^{-6} M dodecyl sulfate, either alone or in combination with albumin, exhibited no Ca^{2+}-binding under the conditions employed [7]. The amount of radioisotope adsorbed to the monolayer, Γ, was calculated according to the expression

$$\Gamma = (I - I_b)/SA$$

where I = final radioactivity in cpm, I_b = cpm before the protein was applied, S = specific activity of [^{14}C]ATP, and A = surface area.

RESULTS

Electron microscopy of synaptic membranes

A representative sample of membrane fraction G, prepared from nerve endings of bovine cerebral gray matter is presented in Fig. 1. Most of the material consisted of synaptic membranes with a few small vesicular components and larger membranous fragments possibly derived from myelin and axonal processes. The other membranous fractions (electron micrographs not shown) obtained after hypotonic shock of the crude pellet contained the following: D, mostly myelin with some glialaxonal processes; E, myelin+processes+synaptic membranes; F, large synaptic membranes+processes; G, mostly synaptic membranes+small processes; H, mostly synaptic membranes+mitochondrial fragments+small processes; I, mostly mitochondria, intact and fragmented. The appearances of the various subcellular components are similar to those obtained from rat brain and described elsewhere [6].

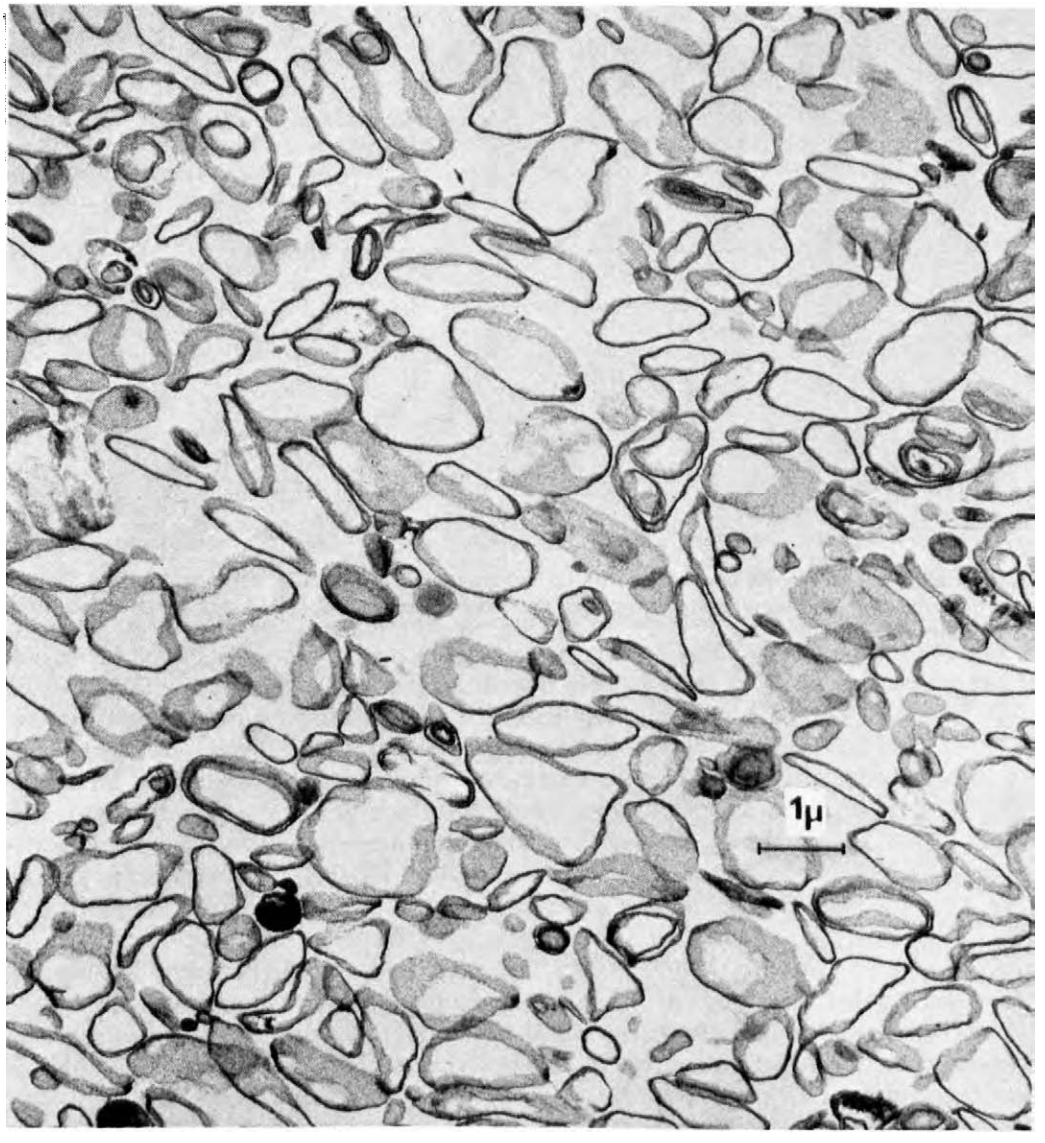

Fig. 1. Electron micrograph of synaptic membranes (layer G) isolated from bovine cerebral gray matter. Specimens prepared as described elsewhere [6].

Gel electrophoresis patterns of the various subcellular fractions

A comparison was made of the gel electrophoretic pattern of the various components of the nerve ending fraction of bovine brain after hypotonic shock and separation by sucrose density gradient centrifugation (Fig. 2). The patterns for fractions F–H, which consisted largely of various membranous fragments of nerve endings, were quite similar. Fractions H and I, which contained mitochondria and intact or partially disrupted nerve endings, were similar to one another. The pattern for the myelin fraction, D, was distinct from the others.

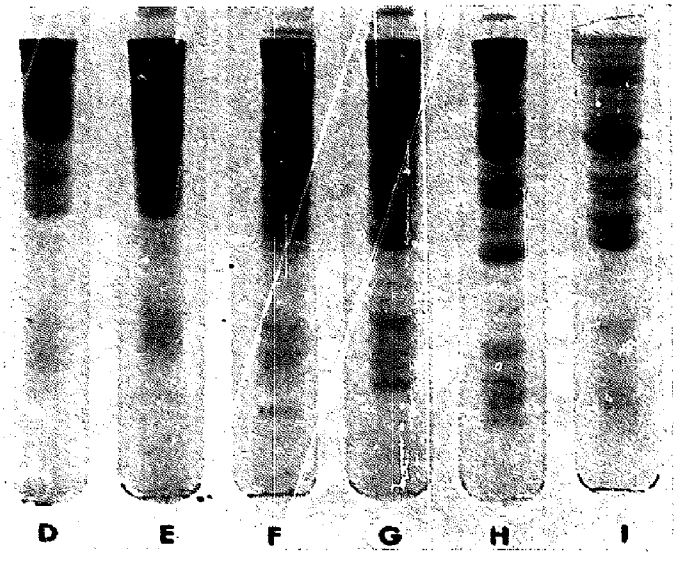

Fig. 2. Acrylamide gel electrophoretic patterns of various membranous fractions obtained from a crude synaptosome-mitochondria pellet from bovine brain. See text for description of subcellular nature of various components.

Fig. 3. Preparative acrylamide gel of bovine synaptic membranes. The pattern is from membrane fraction G; approximately 5 mg of protein per gel. The regions are numbered I–IX.

TABLE I

PERCENT DISTRIBUTION, N-TERMINAL AMINO ACIDS, AND MOLECULAR WEIGHT OF VARIOUS HYDROPHOBIC PROTEINS OF BOVINE SYNAPTIC MEMBRANES

The major end group is indicated by italics, with the additional amino acids representing minor components. The determinations are based on 2 independent preparative gel runs; and in each case matched segments from 6 gels were combined. The agreement in gel patterns and end group analysis between the two was very good.

Protein	Total protein (%)	N-terminal amino acid	$M_r \cdot 10^{-4}$
I	7.1	Ser, Gly	10–25
II	10.6	Ser, Gly, Met	8.5
III	16.4	Ser, Gly, Phe	5.8
IV	29.1	Gly, Met, Ser	5.2
V	14.6	Gly, Met	3.9–4.4
VI	20.0	Gly, Met	3.2–3.3
VII	1.9	Ser, Gly	2.3
VIII	2.0	Ser, Met	2.0
IX	6.0	Met, Ser	1.6
Synaptic membrane	100.0	Ser, Gly, Phe, Met, Ala, Pro	

Since fraction G appeared to consist mainly of synaptic membranes and was the most homogeneous of the fractions, it was selected for preparative gel electrophoresis. The preparative gel was sectioned into 9 bands (I–IX) from which the various protein components were extracted as described earlier (Fig. 3). Most of the bands contained one major and one or two minor components, except VII–IX, which appeared to be single components. A percentage distribution of the total synaptic membrane protein, among the individual protein fractions is presented in Table I. About 60% of the total protein was associated with fractions III–IV, having molecular weights ranging from $5.8–3.2 \cdot 10^4$. The Ca^{2+}-binding protein, IX, comprised only 6% of the total protein and had a molecular weight of $1.6 \cdot 10^4$.

N-terminal amino acids

The major N-terminal amino acid found in fractions I–III was serine, with lesser amounts of glycine in II and III and a trace of phenylalanine in III (Table I). Fractions IV–VI contained mainly glycine with lesser amounts of methionine; in addition there was a trace of serine in IV. Fractions VII and VIII consisted mainly of serine with small amounts of glycine and methionine respectively. Methionine was the major component of IX with a lesser amount of serine. In the total protein two additional end groups, alanine and proline, were detected.

Amino acid composition of proteins

The amino acid composition was performed on the total membrane proteins as well as some of the fractions separated by gel electrophoresis (Table II). Among the significant differences between the various proteins is the relatively low percentage of acidic amino acids in VI (16%) as compared to I and IX (over 26%). Another difference is the high percentage of serine and low percentage of alanine in I. Protein IX had the highest amount of acidic and by far the least amount of basic amino acids. In addition to having a high percentage of acidic amino acids, IX had an unusually

TABLE II

AMINO ACID COMPOSITION OF VARIOUS HYDROPHOBIC PROTEINS

Glutamic acid includes glutamine and aspartic includes asparagine. Tryptophan was not determined. Half-cystine was not determined for III, IV, and Pt_9. The total proteins is expressed as mean ±S.D.; all other data represents an average of 2–3 separate determinations with an agreement of less than 8 %. n.d., not determined.

	Total protein	I	III	IV	VI	IX	Pt_9
Asp	9.9±0.2	10.3	10.1	10.6	7.6	11.6	9.7
Thr	5.5±0.2	5.3	6.1	6.2	6.7	7.3	11.2
Ser	6.6±0.6	11.4	7.5	8.1	8.6	9.3	9.8
Glu	12.8±0.4	16.3	11.2	10.7	8.5	14.7	10.6
Pro	5.6±0.3	6.4	4.5	6.2	3.9	1.2	0
Gly	7.5±0.2	9.7	8.8	9.1	10.1	12.1	15.3
Ala	8.3±0.2	3.6	10.8	12.0	12.8	9.4	12.1
Half Cys	0.8±0.3	0.6	n.d.	n.d.	0.8	0.7	n.d.
Val	6.0±0.1	4.1	7.0	6.9	4.8	7.4	4.8
Met	2.1±0.1	1.8	1.5	1.4	1.0	0.8	0
Ile	4.3±0.5	2.7	4.9	4.3	4.2	5.3	6.2
Leu	8.6±0.2	12.1	9.8	9.9	10.1	9.9	5.5
Tyr	3.1±0.1	0.9	3.2	2.8	3.4	2.3	1.1
Phe	3.8±0.2	6.5	4.3	4.0	5.3	3.7	1.6
Lys	7.6±0.4	5.0	5.0	3.9	6.1	2.4	0
His	2.3±0.1	1.4	2.2	1.6	2.7	0.8	4.5
Arg	5.0±0.2	4.6	4.0	2.8	4.5	1.8	4.5
Gly+Asp	22.7	26.6	21.3	21.3	16.1	26.3	20.3
Lys+His+Arg	14.9	11.0	11.2	8.3	13.3	4.0	9.0
Hydrophobic	31.2	34.4	33.6	34.9	28.4	30.5	14.3

small amount of basic amino acids. Pt_9, the tryptic peptide derived from IX, was comprised of 20 % acidic amino acids and 9 % basic, equally divided among histidine and arginine.

Peptide maps of tryptic digests

Peptide mapping was performed on 6 of the proteins (II–VI, IX) separated by preparative gel electrophoresis (Fig. 4, a–f). There were some similarities between II and III, as well as between IV and V. Both II and III contained more cathodally migrating (basic) peptides than did the other proteins. All the proteins consisted of anodally migrating (acidic) components. Other than a few large acidic peptides, IX was almost devoid of any other components. Methyl green, which was used as a marker, had a mobility similar to arginine, moving to the extreme left edge of the plate.

Ca^{2+}-binding to various proteins and acidic peptide of synaptic membranes

Ca^{2+}-binding was determined on the protein fractions separated by preparative gel electrophoresis (Table III). The highest specific activity was found in the lowest molecular weight component, IX, with the next highest activity in VII and I. Some Ca^{2+}-binding was detectable in all the remainder proteins. The acidic peptide, Pt_9, was found to have a specific activity which was twice that of the original protein IX from which it was derived.

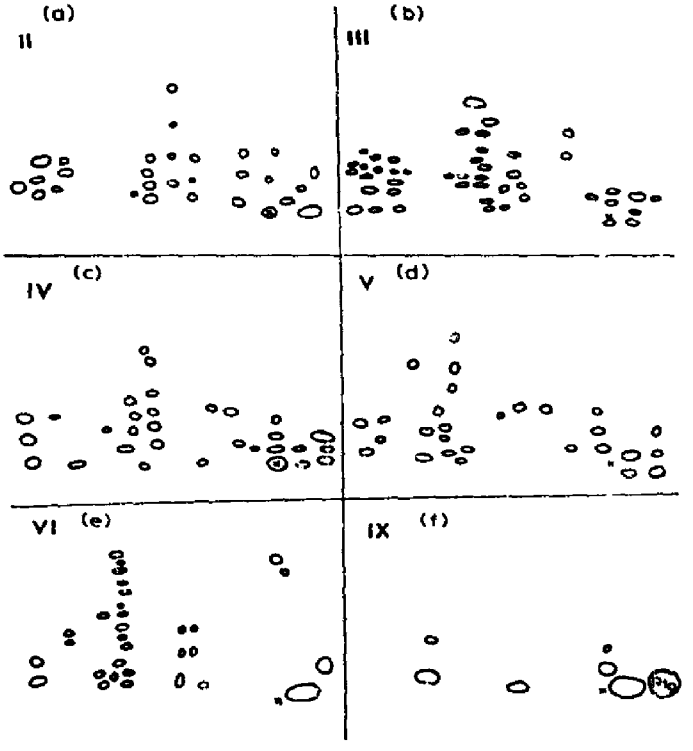

Fig. 4. (a–f) Peptide mapping of tryptic digests of various hydrophobic proteins of bovine synaptic membranes. The proteins are indicated by the roman numerals. Electrophoresis was in the horizontal direction (cathode at left) and chromatography in the vertical direction. The origin is indicated by "×".

TABLE III

Ca^{2+} AND ATP BINDING TO VARIOUS HYDROPHOBIC PROTEINS OF BOVINE SYNAPTIC MEMBRANES

Each value is an average of determinations made of 3 separate protein preparations and the agreement was within 10 % of the mean.

Protein	Ca^{2+} adsorption		ATP adsorption
	Ultrafiltration method (nmol/mg)	Surface film method (nmol/mg)	Surface film (nmol/mg)
Total membrane	125	250	42
I	120	295	48
II	80	125	33
III	65	58	10
IV	22	65	25
V	24	32	3
VI	8	25	4
VII	75	90	2
VIII	150	170	2
IX	220	240	1
VII+VIII+IX	295	305	0
Pt_9	550	—	0

When the Ca^{2+}-binding activity of the various proteins was determined by the surface film technique somewhat different results were obtained. The greatest adsorption was observed in I, with IX having about 80% of this activity; while the total protein fraction of the membrane exhibited almost the same activity as IX. It is very likely that the difference in Ca^{2+}-binding activity observed with the two methods is due to the fact that the configuration and stability of the interfacial protein film determines the degree of Ca^{2+}-adsorption. As discussed elsewhere [7] the concentrations of the protein solution as well as the detergent or organic solvent used to disperse the protein must be optimal for maximal Ca^{2+}-adsorption and film stability. With the ultrafiltration technique the proteins probably exist in the form of aggregates, while the availability of the acidic sites on the protein may be more constant and not susceptible to such interfacial factors as energy barriers and steric configuration [7]. It is conceivable, however, that the interfacial phenomena are, to some extent, representative of the situation in the intact biomembrane; and for this reason it seems appropriate to include data obtained by both methods.

K_m of Ca^{2+}-binding to IX

The adsorption of Ca^{2+} to an interfacial film of IX increased linearly from 10^{-6} to $5 \cdot 10^{-4}$ M Ca^{2+}, and was constant thereafter (Fig. 5). The K_m was estimated at $1.5 \cdot 10^{-5}$ M. At saturation ($5 \cdot 10^{-4}$ M Ca^{2+}) the ratio of molecules of Ca^{2+} bound to molecules of protein was about 4. The pH optimum Ca^{2+}-binding to IX was 8.0, the curve (data not shown) being similar to that obtained previously for a cruder preparation of hydrophobic proteins [7].

ATP-binding

ATP-binding was found to be mainly associated with the higher molecular weight components in addition to the total membrane proteins (Table II). The greatest binding was associated with I, while II had about 2/3 and IV about 1/2 of the activity of I. None of the components with molecular weights below $5 \cdot 10^4$ exhibited ATP-binding.

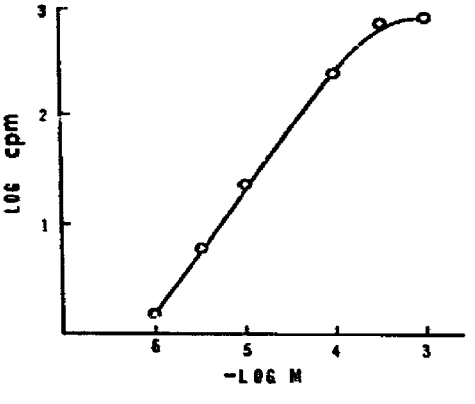

Fig. 5. Ca^{2+}-adsorption curve for the low molecular protein. $^{45}Ca^{2+}$-binding was measured by the surface film technique. Ordinate, log of radioactive cpm; abscissa, molar concentration of $CaCl_2$ adjusted to pH 7.5 with NaOH.

DISCUSSION

Although there appears to be more than one Ca^{2+}-binding protein among the numerous hydrophobic proteins present in synaptic membranes, the lower molecular component examined in the present study exhibits the greatest extent of binding. Approximately 4 Ca^{2+} are bound per molecule of protein, assuming a M_r of 16 000. Since the binding is electrostatic [7] and since there are 14 residues of aspartate and 16 residues of glutamate+glutamine, a sufficient number of acidic residues are available to account for the Ca^{2+}-binding. Insofar as acidic amino acids comprise a significant percentage of a number of the other hydrophobic proteins isolated, it is not unexpected that they also exhibit Ca^{2+}-binding. The tryptic peptide, Pt_9 which had twice the Ca^{2+}-binding activity of the original protein IX, was comprised of a large percentage of acidic and comparatively few basic amino acids. It is probable that this fragment represents the Ca^{2+}-binding portion of the protein. From the peptide maps of the tryptic digests it can be seen that peptides with comparable electrophoretic mobilities are present in all of the fractions exhibiting Ca^{2+}-binding.

It was found that the combination of three low molecular weight proteins had greater binding activity than any of the individual components. As discussed elsewhere [7] the Ca^{2+}-binding occurs within hydrophobic regions of the proteins; consequently it is conceivable that the combination of different proteins could help stabilize the Ca^{2+}-binding by providing favorable ion-dipole interactions, involving, for example, tyrosine or serine-OH groups [7]. In this respect it should be noted that the total protein fraction as well as the intact synaptic membranes [7] exhibited Ca^{2+}-binding.

Some discussion is necessary as to why more emphasis was placed on fraction IX than on I or other components. One of the main reasons is that fraction IX was more readily obtainable from preparative gel electrophoresis in a purer form. Because of its comparatively low molecular weight it was expected to be more readily analyzable and soluble in aqueous media. Preliminary attempts to solubilize IX in various aqueous media has, however, proved unsuccessful. Finally, the relative concentration of Pt_9, which may be a common component of the various Ca^{2+}-binding fractions, is highest in fraction IX. Although fractions I or II are of interest, they are considerably more difficult to remove from the gels, while their relative concentration is much less than IX.

Little can be said about the functional significance of the Ca^{2+}-binding protein. Ca^{2+} is known to play a role in the release and storage of neurotransmitters found in nerve endings as well as with the process of membrane depolarization [13]. Since the preparation used contained both presynaptic and postsynaptic membranous components, it is not known whether the Ca^{2+} protein is derived from only one or both components. Since Ca^{2+}-binding was found in a number of the proteins isolated, the question arises as to whether a single protein, present either as a contaminant or polymeric unit, may be responsible. It is unlikely that the component was a contaminant, since the acrylamide patterns and chemical data were reproducible. The possibility exists, however, that some of the components existed in the form of polymers or aggregates. Although the initial 2% concentration of dodecyl sulfate used for solubilizing the protein prior to gel electrophoresis was sufficient to prevent aggregation or polymerization, the concentration decreased rapidly (to 0.2%) as the protein migrated into the gel. As indicated by the N-terminal amino acid analysis as well as

the electrophoretic patterns, none of the protein fractions were homogeneous; however, all contained one major homogeneous component. The acrylamide gel pattern obtained with the protein mixture was dependent on the concentration of dodecyl sulfate used. If, for example, the concentration of dodecyl sulfate used for solubilization was increased 2–3 times, the resolution of the proteins was markedly decreased.

Although some of the hydrophobic proteins, particularly the lower molecular weight components, exhibit the greatest activity, Ca^{2+}-binding to varying degrees appears to be a characteristic of all the individual proteins of synaptic membrane. Furthermore, the total protein as well as intact membrane exhibit a high degree of Ca^{2+}-binding. Of particular significance is the fact that the various peptide maps presented would appear to have a tryptic peptide similar to Pt_9, a finding which suggests the possibility of an acidic peptide chain common to a number of the proteins exhibiting Ca^{2+}-binding. ATP-binding, on the other hand, is apparently associated with the higher molecular weight components ($1–3 \cdot 10^5$) and a component with a molecular weight of $5 \cdot 10^4$. Since the pH curve had a sharp maximum at pH 4.0 with no binding at pH 6.0, it would appear that basic amino acids such as lysine, arginine and histidine were involved in ATP-binding. From the peptide maps of proteins I, III, and IV, all showing significant ATP-binding, it can be seen that there are numerous tryptic peptides in the basic (cathodal) region. In those proteins with few basic peptides (e.g. VI and IX), there is little or no ATP-binding. In a previous study analyzing the amino acid composition of peptides obtained from the total hydrophobic protein of bovine synaptic membranes, a basic peptide with 42 % basic amino acids was believed to be responsible for the ATP-binding [14].

With respect to ATP-binding, the biological implications are more difficult to assess, since the binding is only demonstrable at a pH below 6 [6]. As discussed elsewhere [6] it is possible for the pH within the ionic diffuse double of a membrane interface to be 1–2 pH units higher than the surrounding medium [17]. Insofar as isolated synaptosomes and synaptic membranes contain the highest concentration of ATP demonstrable in organelles derived from neural tissue [6], the binding of ATP to the hydrophobic proteins may be indicative of a storage mechanism. ATP is believed to be involved in the storage and release [15, 16] of neurotransmitters from synapses, as well as in the binding of Ca^{2+} [18].

It should be recognized that none of the proteins isolated by the present procedures are pure, as indicated by both the end groups analysis as well as by the gel electrophoretic patterns. All attempts to further purify the proteins by gel filtration (Sepharose) have been unsuccessful, largely because of their insolubility in the absence of dodecyl sulfate or other detergents. Another important consideration is the extent to which exposure to dodecyl sulfate during the preparative procedure may have altered the structural and functional properties of the individual proteins. As discussed elsewhere [14] most of the enzymic activity, such as $(Na^+ + K^+)$-ATPase, is destroyed by the treatment, but Ca^{2+}- and ATP-binding are not. Furthermore, structural alterations of the proteins have resulted from the extensive treatment which have undoubtedly altered normal functional and binding characteristics. On the other hand, after solubilization of the membrane proteins in dodecyl sulfate, and subsequent removal of the detergent, the proteins retain their ability to reform vesicular and lamellar structures resembling those of natural membranes [14].

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