Partial Purification of Molecular Weight 12000 Fatty Acid Binding Proteins from Rat Brain and Their Effect on Synaptosomal Na^+-Dependent Amino Acid Uptake†

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ABSTRACT: High-affinity, Na^+-dependent synaptosomal amino acid uptake systems are strongly stimulated by proteins which are known to bind fatty acids, including the M_r 12000 fatty acid binding protein (FABP) from liver. To explore the possibility that such a function might be served by fatty acid binding proteins intrinsic to brain, we examined the 105000*g* supernatant of brain for fatty acid binding. Observed binding was accounted for mainly by components excluded by Sephadex G-50, and to a small degree by the M_r 12000 protein fraction (brain FABP fraction). The partially purified brain FABP fraction contained a protein immunologically identical with liver FABP as well as a FABP electrophoretically distinct from liver FABP. Brain FABP fraction markedly stimulated synaptosomal Na^+-dependent, but not Na^+-independent, amino acid uptake, and also completely reversed the inhibition of synaptosomal Na^+-dependent amino acid uptake induced by oleic acid. Palmitic, stearic, and oleic acids were endogenously associated with the brain FABP fraction. These data are consistent with the hypothesis that M_r 12000 soluble FABPs intrinsic to brain may act as regulators of synaptosomal Na^+-dependent amino acid uptake by sequestering free fatty acids which inhibit this process.

Isolated nerve ending particles (synaptosomes) contain high-affinity, Na^+-dependent amino acid uptake systems which are considered specific for neurotransmitter amino acids such as glycine, glutamic acid, and γ-aminobutyric acid (GABA)[1] (Peterson & Raghupathy, 1972; Levi & Raiteri, 1973; Bennett et al., 1973; Snyder et al., 1973). These Na^+-dependent synaptosomal amino acid uptake systems are specifically inhibited by low concentrations of unsaturated but not saturated fatty acids (Rhoads et al., 1982a). Free fatty acids are present in synaptosomes (Rhoads et al., 1983a), presumably as a result of lipase action on endogenous substrates such as phospholipids (Bazan, 1971; Woelk & Porcellati, 1973).

It has been shown that these Na^+-dependent transport systems are stimulated by several proteins which share the common feature of binding free fatty acids and which on incubation with synaptosomes diminish their free fatty acid content (Rhoads et al., 1983a). Thus, stimulation of synaptosomal Na^+-dependent amino acid transport was found with bovine serum albumin, hepatic fatty acid binding protein (FABP), β-lactoglobulin, and α-fetoprotein but not with ribonuclease, casein, gelatin, or trypsin inhibitor. In addition, the portion of the albumin molecule responsible for the stimulation (Raghupathy et al., 1978) was shown to comprise the same sequence (residues 377–504) previously implicated in the binding of long-chain fatty acids (Reed et al., 1975).

These observations have led to the suggestion that fatty acids are potential regulators of neuroactive amino acid transport in synaptosomal systems, possibly exerting their effects via interaction with fluid domains in the synaptosomal membrane (Rhoads et al., 1982b, 1983a). Endogenous fatty acid binding proteins may thus play an important role in the synaptosomal amino acid transport process by virtue of their effect on these domains, consequent to the removal of free fatty acids from them. At least two distinct M_r 12000–15000 intracellular fatty acid binding proteins (FABPs) have been isolated and characterized from liver and intestine (Ockner & Manning, 1974; Ockner et al., 1982; Gordon et al. 1983; Alpers et al., 1984), while recently a third distinct M_r 12000 FABP was purified from myocardium (Said & Schulz, 1984).

The purpose of the present study was to ascertain the presence and identity of low molecular weight FABP(s) in brain which might play a role in protecting the Na^+-dependent synaptosomal amino acid transport systems from the inhibitory effects of endogenous free fatty acids.

Experimental Procedures

Materials. Uniformly labeled [^{14}C]oleic acid and amino acids were purchased from New England Nuclear Corp. (Boston, MA); these had the following specific activities (millicuries per millimole): oleic acid, 57; GABA, 203; glutamic acid, 254; aspartic acid, 219; leucine, 298; phenylalanine, 536. Sephadex G-50 was obtained from Pharmacia Fine Chemicals, Inc. (Piscataway, NJ), and all reagents for polyacrylamide gel electrophoresis were from Bio-Rad (Richmond, CA). Membrane filters were obtained from Microfiltration Systems (Dublin, CA). [^{14}C]Methyloleate was prepared by treatment of [^{14}C]oleate with BF_3–methanol (Supelco) and thin-layer chromatography.

Preparation of M_r 12000 Protein Fraction of Brain. All steps were performed at 4 °C. Whole brains were obtained from adult male Sprague-Dawley rats (300–400 g) following decapitation. After being washed in ice-cold 0.01 M potassium phosphate buffer, pH 7.4, and 0.154 M KCl (KCl–phosphate buffer), brains were homogenized in the same buffer (1:1.5 w/v) using a Teflon–glass homogenizer and centrifuged for 20 min at 12000*g*. The resulting supernatant was centrifuged for 1 h at 105000*g*. The high-speed clear supernatant (cytosol) was aspirated in order to minimize contamination by floating fat and was used for subsequent analytical and preparative gel filtration experiments. Cytosol from liver was prepared

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[1] Abbreviations: GABA, γ-aminobutyric acid; FABP, fatty acid binding protein; Tris, tris(hydroxymethyl)aminomethane.

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Table I: Partial Purification of Brain FABP Fraction

fraction	volume (mL)	total protein (mg)	specific binding of [^{14}C]oleic acid to M_r 12000 proteins[b] (nmol/mg of total protein)
105000*g*	38	897	0.047
FABP fraction[a]	1	3.9	1.37

[a] The M_r 12000 FABP fraction was partially purified from brain 105000*g* supernatant by two consecutive Sephadex G-50 gel chromatography steps as described under Experimental Procedures. [b] Specific binding of [^{14}C]oleic acid to M_r 12000 proteins was measured in aliquots of the 105000*g* supernatant and brain FABP fraction as described under Experimental Procedures and illustrated in Figures 1 and 2.

in fundamentally the same way (Ockner et al., 1982). The M_r 12000 proteins from rat brain cytosol were prepared as previously described for hepatic cytosol by Sephadex G-50 chromatography in KCl–phosphate buffer (Ockner et al., 1972). Brain cytosol was passed through a 5 × 45 cm column of Sephadex G-50. Fractions of 15 mL were collected. Fractions corresponding to the elution volume of hepatic FABP (M_r 12000 FABP fraction, V_e/V_0 = 1.63–1.83) were pooled, concentrated to 2.5 mL (Amicon YM 5), and passed through a second calibrated Sephadex G-50 column (2.5 × 40 cm). Fractions of 3.5 mL were collected and M_r 12000 protein fractions (V_e/V_0 = 1.63–1.83) collected and pooled. This represented the brain FABP fraction used in subsequent analyses.

Analytical Column Chromatography. Fatty acid binding to aliquots of soluble protein fractions was examined by using a 1 × 40 cm column of Sephadex G-50 equilibrated with KCl–phosphate buffer at 20 °C (Ockner et al., 1980). One to ten milligrams of protein in 0.2–0.4 mL of buffer was loaded after prior incubation for 10 min with 24 nmol (45 nCi) of [^{14}C]oleate. Fractions of 1.1 mL were collected. Protein was measured by the optical density at 280 nm. Eluted radioactivity was counted in 10% Bio-Solv BBS 3 (Beckman Instruments) in Liquifluor (New England Nuclear) and toluene.

Polyacrylamide Gel Electrophoresis. Polyacrylamide disc gel electrophoresis using 7% separating gels at pH 8.9 was performed as previously described (Ockner et al., 1982). Identically run gels either were stained with 0.2% Coomassie brilliant blue or were immediately sectioned into 1.8-mm slices. Radioactivity in gel slices was determined in 10 mL of Liquifluor–toluene after partial hydrolysis of the gel in NCS (Amersham) by the method of Grower & Bransome (1970).

Immunochemical Studies. Specific antisera to rat liver FABP and rat intestinal FABP were prepared as previously described (Ockner & Manning, 1974; Ockner et al., 1982). Immunochemical identity of antigens in various protein fractions was assessed by the Ouchterlony double immunodiffusion method.

Endogenous Fatty Acid Analysis. Lipids were extracted from the M_r 12000 protein fraction by the method of Folch et al. (1957) and were separated into classes by thin-layer chromatography on 0.25 mm of silica gel 60 (EM Laboratories, Inc., Elmsford, NY) employing a solvent system consisting of petroleum ether–diethyl ether–acetic acid (90:15:1.5 v/v). Zones were identified by staining of lipid standards with rhodamine. The free fatty acid zone was then eluted and treated with BF_3–methanol to prepare the methyl esters. The fatty acid methyl esters were separated on a 6-ft column of 10% SP-2330 on 100/120 Chromasorb W AW (Supelco) at 176 °C in a Hewlett-Packard 402B gas chromatograph interfaced with a Hewlett-Packard 3380A digital integrator. Methyl pentadecanoate was employed as an internal standard.

Transport Assays. Synaptosomal (P_2) fractions were prepared from freshly excised cerebral cortices of adult rats essentially as described before (Rhoads et al., 1983b). Synaptosomal fractions (0.05–0.2 mg of protein) were suspended in

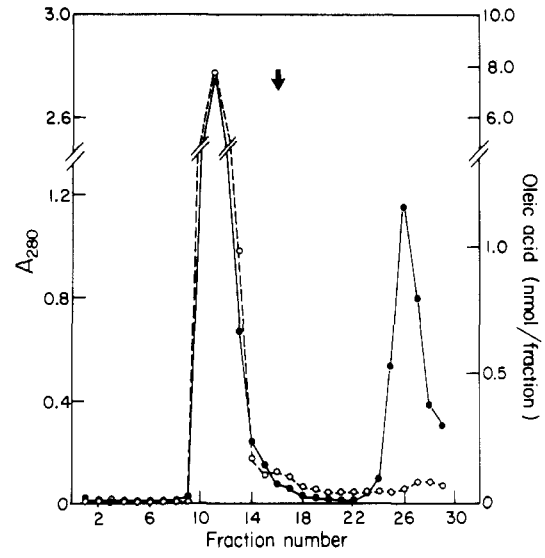

FIGURE 1: Oleic acid binding to rat brain cytosol proteins. Rat brain cytosol (9.4 mg) was incubated with 24 nmol of [^{14}C]oleic acid and chromatographed on a 1 × 40 cm column of Sephadex G-50 as described under Experimental Procedures. (●) Absorbance at 280 nm; (○) oleic acid. The arrow indicates the elution position of pure liver FABP.

1 mL of a medium containing 10 mM Tris-HCl buffer, pH 7.4, 5 mM $MgCl_2$, 3 mM $CaCl_2$, 150 mM NaCl, and 1 mM KCl and incubated either with or without brain M_r 12000 protein fraction (final concentration 0.1 mg/mL) and 0.1 μCi of ^{14}C-labeled amino acids (0.3–0.4 μM) for 10 min at 25 °C. Following incubation, reactions were terminated by the addition of 5 mL of ice-cold buffer, and the synaptosomal particles were harvested on Millipore filters (0.45 μm) and assayed for radioactivity as described previously (Peterson & Raghupathy, 1972).

Miscellaneous Analytical Techniques. Protein concentration was determined by the method of Lowry et al. (1951). Statistical significance of differences was determined by the Student's *t* test.

Results

An aliquot of whole brain cytosol was incubated with [^{14}C]oleate and the pattern of fatty acid binding to protein examined by Sephadex G-50 analytical column chromatography as described under Experimental Procedures. The elution profile of protein and fatty acid is shown in Figure 1. Most of the recovered oleate eluted with the major void volume protein peak (fraction 11). A small peak was consistently observed at the elution volume of M_r 12000 liver FABP (fraction 16), while a third peak of oleate (fraction 27) corresponded to the elution volume of free oleate. The M_r 12000 brain FABP fraction was partially purified from brain cytosol by two-step Sephadex G-50 chromatography as described under Experimental Procedures. The recovery of protein (Table I) and oleate binding to M_r 12000 proteins showed that the brain FABP fraction comprised 0.4% of the total soluble

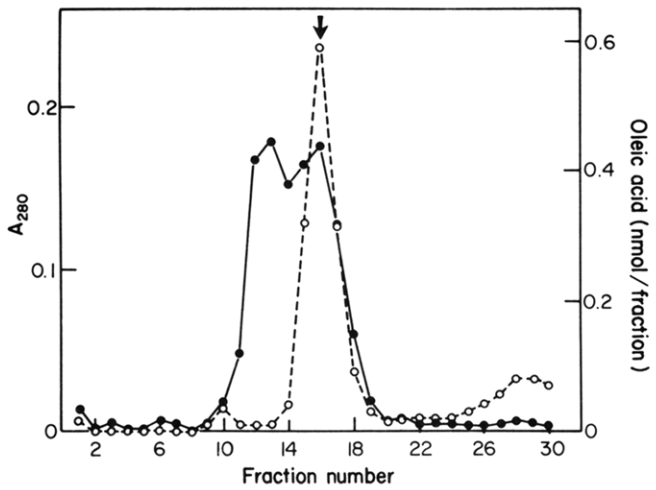

FIGURE 2: Oleic acid binding to rat brain FABP fraction. Rat brain FABP fraction (1 mg) was incubated with 24 nmol of [^{14}C]oleic acid and chromatographed on a 1 × 40 cm column of Sephadex G-50 as described under Experimental Procedures. (●) Absorbance at 280 nm; (○) oleic acid. The arrow indicates the elution position of pure liver FABP.

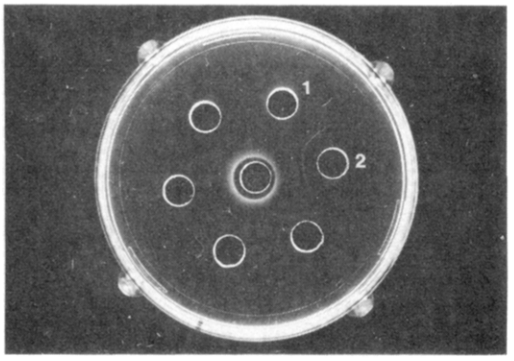

FIGURE 3: Immunodiffusion analysis of rat brain FABP fraction. Well 1 contains 12 μg of rat liver cytosol; the center well contains 158 μg of rat brain FABP fraction; well 2 contains rabbit antiserum to rat liver FABP.

protein of brain and was enriched approximately 30-fold by gel chromatography. The pattern of oleate binding to an aliquot of FABP fraction of brain cytosol on analytical column chromatography is shown in Figure 2. Three peaks of oleate were again observed, the peak coinciding with the elution volume of liver FABP being the most prominent. Substitution of NaCl for KCl in the elution buffer did not affect the observed pattern of binding. Furthermore, radioactivity recovered from the columns migrated entirely as fatty acid on thin-layer chromatography. These findings suggested that the FABP fraction of brain cytosol contained a protein similar to or identical with rat liver FABP, which had previously been shown to stimulate synaptosomal Na^+-dependent amino acid transport (Rhoads et al., 1983a). The identity and functional properties of the fatty acid binding species in the brain FABP fraction were thus examined further.

Immunodiffusion analysis revealed a single precipitin line of identity between the brain FABP fraction and rat liver cytosol with antiserum to rat liver FABP (Figure 3). FABP comprises 3–5% of hepatic soluble protein. The considerable disproportion between the amounts of liver cytosol protein (12 μg) and brain FABP fraction (158 μg) required to generate the qualitatively similar precipitation reactions seen in Figure 3 indicates that immunologically reactive liver FABP comprises a relatively minor component of the brain M_r 12000 FABP fraction. Indeed, no precipitin reaction was obtained

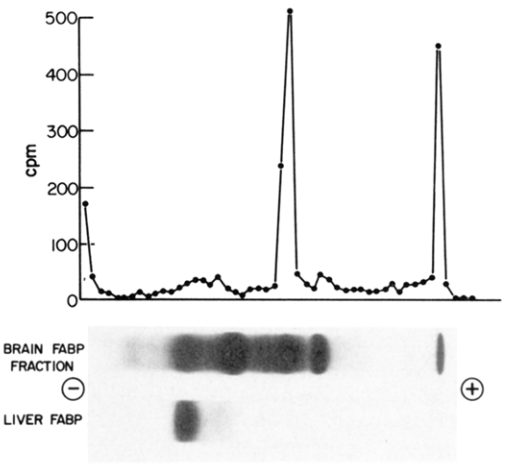

FIGURE 4: Binding of [^{14}C]methyloleate to proteins in brain FABP fraction on polyacrylamide gel electrophoresis. Brain FABP fraction (300 μg) was incubated with 47 pmol (2.7 nCi) of [^{14}C]methyloleate, separated by polyacrylamide gel electrophoresis, and counted as described under Experimental Procedures. An identical gel was stained with Coomassie blue and is shown as the top gel; the bottom gel was run with 10 μg of pure rat liver FABP (shown for comparison).

Table II: Effect of Brain FABP Fraction on Synaptosomal Uptake of Amino Acids

^{14}C-labeled amino acid	uptake[a] (pmol/mg of protein)		FABP effect (% of control)	P
	control	+FABP		
γ-aminobutyric acid (4)	1110 ± 197	1470 ● 86	132	<0.02
glutamic acid (4)	2170 ± 219	3200 ± 110	152	<0.01
aspartic acid (3)	3490 ± 366	4460 ± 260	128	<0.01
leucine (4)	82 ± 10	79 ± 8	96	NS[b,c]
phenylalanine (3)	89 ± 6	82 ± 7	92	NS[b,c]

[a] Synaptosomal fractions were incubated for 10 min with the ^{14}C-labeled amino acids in the presence or absence of brain FABP fraction as described under Experimental Procedures. Values given represent the mean ± SEM of the number of experiments (given in parentheses). Brain FABP fraction was used at a concentration of 0.1 mg/mL. [b] NS, not significant. [c] Lack of effect of FABP in the uptake of these amino acids was seen in both Na^+-containing and Na^+-free incubation media.

with whole brain cytosol and antiserum to rat liver FABP. This contrasts with liver, in which FABP comprises up to 80% of the M_r 12000 protein fraction (N. M. Bass, unpublished results). Furthermore, the proteins associated with the peak of oleate binding in the void volume of brain cytosol chromatographed on Sephadex G-50 (Figure 1) showed no reaction with antiserum to liver FABP. Antiserum to intestinal FABP gave no reaction with up to 1000 μg of brain FABP fraction (data not shown).

To further determine the nature of the fatty acid binding component of the brain FABP fraction, the binding of [^{14}C]methyloleate to proteins in this fraction was examined by disc gel electrophoresis. As shown in Figure 4, two main peaks of [^{14}C]methyloleate were detected; one was associated with the bromophenol blue front; and other, comprising 34% of recovered methyl oleate, was associated with a protein band migrating more anodally than liver FABP (shown for comparison, Figure 4). Although a protein band was seen in the M_r 12000 FABP fraction of brain with migration identical with that of liver FABP, radioactivity associated with this band was slight in comparison to the more anodal species.

The effect of brain FABP fraction on Na^+-dependent synaptosomal accumulation of amino acids was examined at a protein concentration of 0.1 mg/mL. As shown in Table II, brain FABP fraction stimulated only the sodium-dependent

Table III: Endogenous Fatty Acid Content of Brain FABP Fraction

	fatty acid content (nmol/mg of protein)[a]			
expt	palmitic acid (16:0)	stearic acid (18:0)	oleic acid ($18:1^{\Delta 9}$)	total[b]
1	1.27	0.51	0.75	2.53
2	3.45	0.69	0.63	4.77

[a] Fatty acids were extracted from 1–3.4 mg of brain FABP fraction protein and the fatty acid methyl esters analyzed on gas–liquid chromatography as described under Experimental Procedures. Values from two separate experiments using two different preparations of brain FABP fraction are given. [b] Linoleic ($18:2^{\Delta 9,12}$), linolenic ($18:2^{\Delta 9,12,15}$), and arachidonic ($20:4^{\Delta 5,8,11,14}$) acids were not detected.

uptake of GABA, glutamic acid, and aspartic acid, while uptake of leucine and phenylalanine, which proceeds via Na^+-independent mechanisms, was unaffected. Furthermore, when oleic acid (15 μM) was added to assays of Na^+-dependent glutamic acid transport, marked inhibition of uptake occurred (53% of control). This inhibition by oleic acid was completely reversed (108% of control) by the addition of brain FABP fraction at a concentration of 0.1 mg/mL. Since these measurements were made at 10 min, a time at which amino acid accumulation is approaching equilibrium (Rhoads et al., 1983a), we conducted additional experiments at 3 min. Both the stimulatory effect of brain FABP fraction as well as its reversal of the inhibitory effect of oleic acid on Na^+-dependent amino acid uptake were present at this earlier time point (data not shown). Since the proposed mechanism whereby fatty acid binding proteins stimulate Na^+-dependent neuroactive amino acid transport involves the sequestration of endogenous fatty acids (Rhoads et al., 1983a), we measured the fatty acids associated with the M_r 12000 FABP fraction. As shown in Table III, , palmitic acid (16:0), stearic acid (18:0), and oleic acid ($18:1^{\Delta 9}$) were detected in association with the FABP fraction. The total free fatty acid content of the FABP fraction was relatively small, comprising 2.5–4.8 nmol/mg of protein.

Discussion

Previous studies have demonstrated a stimulatory effect of several fatty acid binding proteins on synaptosomal Na^+-dependent amino acid uptake which appeared to be mediated through the removal of free fatty acid inhibition of this transport process (Rhoads et al., 1983a). Included among the proteins displaying this effect was the intracellular M_r 12000 liver FABP. Several immunologically and structurally distinct intracellular FABPs have been isolated from a variety of tissues, including liver (Ockner et al., 1982), small intestinal mucosa (Ockner & Manning, 1974), and heart (Said & Schultz, 1984), all of approximately the same size (M_r 12000). Furthermore, several additional proteins in this size range are committed to binding specific hydrophobic ligands including retinol and retinoic acid (Chytil & Ong, 1979), sterols (Noland et al., 1980; Dempsey et al., 1981), and phospholipids (Bloj & Zilversmit, 1977). Substantial sequence homology exists between different FABPs (Alpers et al., 1984) as well as among FABPs and other lipid binding proteins including the P_2 protein associated with peripheral nerve myelin (Takahashi et al., 1982). We were therefore interested in determining whether proteins identical with or similar to previously characterized FABPs existed in central neural tissue, which may be of physiological importance in terms of regulating the Na^+-dependent transport of neuroactive amino acids. Our studies with brain cytosol revealed extensive binding of radiolabeled oleic acid to relatively high molecular weight proteins in addition to binding to M_r 12000 proteins (brain FABP fraction). The binding of oleic acid to the void volume proteins of brain cytosol was not examined in detail in this study but merits some comment. This peak of oleate binding did not react with antiserum to liver FABP and may represent nonspecific (nonsaturable) binding of oleic acid by one or more proteins in the relatively high molecular weight range of soluble proteins in a manner similar to that described for hepatic cytosol (Ockner et al., 1980). However, Fournier et al. (1983) have recently shown that heart muscle FABP may exist in polymolecular states, and the possibility that the brain FABP, which was shown to be electrophoretically distinct from liver FABP (Figure 4), may behave in the same fashion must be considered as a possible explanation for the observed binding to the void volume proteins in brain cytosol. Further studies will be required to resolve this important issue.

Our findings show that in contrast to liver, where hepatic FABP comprises 3–5% of total soluble protein, the brain FABP fraction (which contains several proteins) accounts for approximately 0.4% of the total soluble protein of whole brain. If we assume (1) that the protein or proteins which are responsible for the observed fatty acid binding within the heterogeneous M_r 12000 brain FABP fraction possess a single, saturable binding site for fatty acids (Mishkin & Turcotte, 1974; Tipping et al., 1975; Glatz & Veerkamp, 1983) and (2) that the binding of endogenous fatty acids (Table III) represents full occupancy of these binding sites (2.5–4.8 nmol/mg of protein in the main FABP fraction), then it can be inferred that the specific low molecular weight FABP of brain comprises between 30 and 60 μg/mg (3–6%) of the brain FABP fraction. However, as discussed later, this relatively low apparent concentration of M_r 12000 FABP in brain may be sufficient to achieve a physiologically important role.

We examined the possibility that brain FABP might be immunologically related to liver FABP and/or intestinal FABP. Immunological identity was indeed found between liver FABP and a component of the brain FABP fraction, whereas no reaction was detected with antiserum specific for intestinal FABP. Although the radial immunodiffusion technique employed in these studies is essentially qualitative, our findings indicated that the immunologically reactive hepatic FABP-like component in brain FABP fraction was present in a quantity clearly less than could account for the amount of observed binding of either exogenous or endogenous fatty acids. This suspicion was further borne out by the finding that [^{14}C]-methyloleate binding by brain FABP fraction was most marked in relation to a protein showing different electrophoretic behavior from hepatic FABP. The nature of this protein is unknown, but it is clearly different from both intestinal and hepatic FABP. Since antiserum to heart FABP is not available, we were unable to test for an immunological relationship between this protein and the distinct brain FABP. The important possibility that such a relationship may exist is supported first by the findings of Lee & Wiggert (1984), who recently identified an M_r 14800 FABP from the cytosol of chick neural retina which was also present in cardiac muscle but not in liver, and which thus also appears to be distinct from the FABP present in avian liver (Levine et al., 1971). Second, the M_r 12000 mammalian cardiac FABP appears to be a faster migrating protein on gel electrophoresis under basic pH conditions than is either liver or intestinal FABP (Ockner & Manning, 1974; Ockner et al., 1982; Said & Schultz, 1984). Further purification of the new binding protein identified in the brain FABP fraction will be needed to examine these possibilities.

The results of these studies with brain FABP fraction on synaptosomal Na^+-dependent amino acid transport extend

previous observations on the similar effects of other fatty acid binding proteins (Rhoads et al., 1983a) and support a regulatory role for brain FABP in this activity of synaptosomes. Brain FABP fraction comprises approximately 0.4% of brain cytosolic protein. Assuming that the in vivo protein concentration in brain cytosol is approximately 60 mg/mL, it is clear that the concentration of brain FABP fraction employed in these studies (0.1 mg/mL) approximates it in vivo concentration. Stimulation of synaptosomal Na^+-dependent uptake of GABA, glutamic acid, and aspartic acid was observed to 128–152% of control values, while the Na^+-independent uptake of leucine and phenylalanine was unaffected. This magnitude of stimulation of Na^+-dependent amino acid uptake was previously obtained with hepatic FABP and bovine serum albumin at a concentration of 0.5–1 mg–mL (Rhoads et al., 1983a). However, firm conclusions regarding the potency of brain FABP relative to these other proteins in stimulating synaptosomal amino acid transport cannot be drawn without formal experimental comparison. Of additional significance, brain FABP fractions completely reversed the inhibition of synaptosomal glutamic acid uptake by oleic acid. The inhibition of Na^+-dependent synaptosomal amino acid transport is observed with unsaturated, but not saturated, fatty acids (Rhoads et al., 1982a, 1983a). Maximal inhibition was observed with $18{:}1^{\Delta 6}$ and $18{:}1^{\Delta 9}$ fatty acids. The latter (oleic acid) is present in synaptosomes and is found in the brain FABP fraction as an endogenous ligand. However, it is important to note that the endogenous fatty acid content determined for brain FABP fraction is of the same magnitude as that previously observed in entire synaptosomal extracts (Rhoads et al., 1983a). This apparent lack of enrichment in free fatty acid concentration indicates that brain FABP binds only a small portion of the free fatty acids present in whole synaptosomes. On the other hand, this reasoning assumes that the concentrations of brain FABP present in synaptosomes is similar to that found in whole brain cytosol. It is presently unknown whether this is indeed the case. Thus, although our previous studies (Rhoads et al., 1983a) provided strong evidence in support of a stimulation of synaptosomal Na^+-dependent amino acid transport by fatty acid binding proteins which is effected via the sequestration of endogenous synaptosomal free fatty acids, our present findings suggest the possibility that, in addition to this mechanism, brain FABP fraction may stimulate amino acid transport via other as yet undefined mechanism(s) as well. In any case, our data are clearly in accord with a role for brain FABP in the sequestration of oleic acid which might otherwise partition into the membrane domain of the Na^+-dependent amino acid transporter in synaptosomes [for detailed discussion, see Rhoads et al. (1983a)], leading to inhibition of neuroactive amino acid uptake. In this capacity, brain FABP may protect synaptosomal Na^+-dependent amino acid transport from fluctuations in cerebral free fatty acid levels which may occur in a variety of conditions including ischemia (Bazan, 1970; Shiu et al., 1983; Nemoto et al., 1984), hypoglycemia (Agardh et al., 1981), and electroconvulsive shock administration (Bazan & Rakowski, 1970).

Acknowledgments

We thank Diana Fedorchak and Michael Karasik for help in preparing the manuscript and Dr. Neal Peterson for helpful consultation with these studies.

Registry No. GABA, 56-12-2; glutamic acid, 56-86-0; aspartic acid, 56-84-8; oleic acid, 112-80-1; palmitic acid, 57-10-3; stearic acid, 57-11-4.

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Localization of Binding Sites for Carboxyl Terminal Specific Anti-Rhodopsin Monoclonal Antibodies Using Synthetic Peptides†

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ABSTRACT: The binding sites for four monoclonal antibodies, rho 1D4, rho 3C2, rho 3A6, and rho 1C5, have been localized within the C-terminal region of bovine rhodopsin: Asp[18′]-Glu-Ala[16′]-Ser-Thr-Thr-Val[12′]-Ser-Lys-Thr-Glu[8′]-Thr-Ser-Gln-Val[4′]-Ala-Pro-Ala[1′]. Antibody binding sites were localized by using synthetic C-terminal peptides in conjunction with solid-phase competitive inhibition assays and limited proteolytic digestion of rhodopsin in conjunction with electrophoretic immunoblotting techniques. Binding of the rho 1D4 and rho 3C2 antibodies to immobilized rhodopsin was inhibited with peptides of length 1′–8′ and longer. Antibody rho 1D4 binding was not inhibited by peptides 2′–13′ or 3′–18′, indicating that the C-terminal alanine residue of rhodopsin was required. Similar competitive inhibition studies indicated that the antibody rho 3A6 required peptides of length 1′–12′ and longer whereas rho 1C5 required peptide 1′–18′. Peptide 3′–18′ was as effective as 1′–18′ in inhibiting rho 3A6 binding to rhodopsin, but replacement of glutamic acid in position 8′ with glutamine abolished competition. This substitution had little effect on the binding of antibody rho 1C5. Thus, Glu[8′] was essential for rho 3A6 binding but not for the binding of the rho 1C5 antibody. Cleavage of the seven amino acid C-terminus from rhodopsin and further cleavage to F_1 (M_r 25 000) and F_2 (M_r 12 000) fragments with *Staphylococcus aureus* V8 protease abolished binding of rho 1D4 antibody to the membrane-bound rhodopsin fragments. Antibodies rho 3A6 and rho 1C5, however, were found to bind both rhodopsin lacking the seven amino acid C-terminus and the corresponding F_2 fragment. These results indicate that a peptide longer than 1′–6′ is required for antibodies rho 1D4 and rho 3C2 binding, 8′–12′ for antibody rho 3A6 binding, and 9′–17′ or 9′–18′ for antibody rho 1C5. Since longer peptides and rhodopsin are more effective competitors, other factors such as conformation also affect binding reactivity. These studies in conjunction with related studies on the binding of these antibodies to membrane-bound and detergent solubilized bleached and unbleached rhodopsin indicate that the carboxyl-terminal 1′–18′ segment of rhodopsin is highly accessible to these immunological probes and relatively insensitive to the state of bleaching and solubilization of rhodopsin. The cross-reactivity of these monoclonal antibodies as well as monoclonal antibodies against other regions of bovine rhodopsin was also studied. Results indicate that the C-terminal segment (as specified by the rho 1D4 and rho 3C2 antibodies), the F_1–F_2 linking region, and the N-terminal region of rhodopsin are highly conserved in pig, dog, cat, rat, rabbit, and frog rhodopsins. Segments farther in from the C-terminus as specified by the rho 3A6 and rho 1C5 antibodies are more variable.

Rhodopsin is the photoreceptor protein of vertebrate rod cells. It consists of a polypeptide chain of M_r 39 000 linked to a molecule of 11-*cis*-retinal [see Hargrave (1982) for a recent review]. The complete covalent sequence of the protein has recently been determined (Ovinchinnikov et al., 1982; Hargrave et al., 1983). Topographic studies carried out in a number of laboratories have shown that the carboxyl-terminal segment of rhodopsin is exposed on the cytoplasmic side of the disk membrane and the amino-terminal segment containing two carbohydrate chains is exposed on the opposite (intradisk) side (Hargrave, 1982). On the basis of numerous labeling and limited proteolysis studies, a model has been constructed in which the polypeptide chain of rhodopsin is visualized as traversing the phospholipid bilayer in seven predominantly hydrophobic, α-helical segments connected by hydrophilic linking regions (Ovinchinnikov et al., 1982; Hargrave et al., 1983).

The carboxyl-terminal segment of rhodopsin has been shown to be highly accessible on the cytoplasmic surface of the disk membrane. A variety of proteolytic enzymes including thermolysin (Hargrave & Fung, 1977), trypsin (Molday & Molday, 1979), *Staphylococcus aureus* protease (Findlay et al., 1981), and papain (Fung & Hubbell, 1978) initially cleave small peptides from the carboxyl terminus. The membrane-impermeable chemical-labeling agent (nitroazidophenyl)taurine also preferentially labels amino acids in the C-terminal region of rhodopsin (Mas et al., 1980). A rhodopsin kinase has also been shown to phosphorylate serine and threonine residues along the C-terminus when rod outer segments (ROS)[1] are bleached in the presence of ATP (Wilden & Kühn, 1982).

More recently, monoclonal antibodies to bovine rhodopsin have been produced and used as probes to study the organi-

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[1] Abbreviations: AUFS, absorbance units full scale; Boc, *tert*-butoxycarbonyl; BSA, bovine serum albumin; CHAPS, 3-[(3-cholamidopropyl)dimethylammonium]-1-propanesulfonate; DCC, dicyclohexylcarbodiimide; HOBt, 1-hydroxybenzotriazole monohydrate; HPLC, high-performance liquid chromatography; Ig, immunoglobulin; PAM, hydroxymethylphenylacetamidomethyl; RIA, radioimmunoassay; ROS, rod outer segments; $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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