this kind may undergo some conformational change(s) to prevent a short circuit.

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Registry No. Glutamic acid, 56-86-0; sodium, 7440-23-5; hydrogen ion, 12408-02-5.

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Modulation of Membrane Transport by Free Fatty Acids: Inhibition of Synaptosomal Sodium-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 free fatty acids. The rate of uptake as well as the overall level of accumulation is increased by such proteins as bovine serum albumin, hepatic fatty acid binding protein, β-lactoglobulin, and fetuin. Such a stimulation is not observed with proteins which do not bind fatty acids. The transport activity of synaptosomal preparations can be directly correlated with the free fatty acid content of the preparation. Thus, incubation with albumin reduces the free fatty acid content of synaptosomal preparations, suggesting that the stimulatory effects of the proteins are related to their removal of inhibitory fatty acids formed by hydrolysis of membrane lipids during incubation. Inhibition of amino acid uptake is seen with most cis-unsaturated long chain fatty acids while saturated and trans-unsaturated fatty acids have relatively little or no effect. Under conditions in which the ionophore gramicidin D causes an increase of ^{22}Na flux into synaptosomes, oleic acid (50 μM) has no effect on the influx. These data are consistent with the hypothesis proposed earlier by us [Rhoads, D. E., Peterson, N. A., & Raghupathy, E. (1982) *Biochemistry 21*, 4782] that Na^+-dependent amino acid transport carrier proteins reside in a relatively fluid lipid domain in the synaptosomal membrane and that the effects of cis-unsaturated fatty acids are mediated by interactions with such domains.

Isolated nerve ending particles (synaptosomes) contain high-affinity, Na^+-dependent amino acid uptake systems which are generally considered to be specific for neurotransmitter amino acids (Peterson & Raghupathy, 1972; Bennett et al., 1973; Snyder et al., 1973). It has been demonstrated that such Na^+-dependent uptake systems are sensitive to the depolarizing agent veratridine (Rhoads et al., 1982b) and thus appear to be linked energetically to sodium ion gradients, a conclusion supported by recent studies on amino acid uptake into plasma membrane vesicles prepared from synaptosomes (Kanner, 1978; Kanner & Sharon, 1978; Mayor et al., 1981; Rhoads et al., 1982c). It was also shown that these Na^+-dependent, veratridine-sensitive transport systems are strongly stimulated by bovine serum albumin (Peterson et al., 1979; Rhoads et al., 1982b). In the case of the transport of one of these amino acids, viz., proline, the portion of the albumin molecule that is responsible for the stimulation was shown to be the region comprising amino acid residues 377–504 (Raghupathy et al., 1978). The same sequence has been previously implicated in the binding of long-chain fatty acids by albumin (Reed et al., 1975). This suggested that the stimulatory effect of serum albumin was related to its ability to bind free (unesterified) fatty acids. Na^+-dependent, synaptosomal amino acid uptake systems were subsequently shown to be specifically inhibited by low concentrations of unsaturated free fatty acids (Rhoads et al., 1982d). Saturated fatty acids had negligible effect on the Na^+-dependent transport systems for proline, glutamic acid, and γ-aminobutyric acid (GABA),[1] while neither satu-

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rated nor unsaturated fatty acids had inhibitory effects on the Na^+-independent transport of amino acids such as phenylalanine and leucine. These results have led to the suggestion that fatty acids are potential regulators of neuroactive amino acid transport in synaptosomal systems. Free fatty acids have been detected in a number of cellular lipid extracts, including extracts from brain preparations (Bazan & Joel, 1968; Bazan, 1970); lipases capable of releasing free fatty acids from endogenous brain substrates such as phospholipids have been partially characterized (Bazan, 1971; Woelk & Porcellati, 1973).

In the present paper evidence is provided that only proteins that have been shown to bind free fatty acids stimulate Na^+-dependent amino acid uptake. Evidence is also provided that free fatty acids are present in synaptosomes, that the synaptosomal free fatty acid content is decreased upon incubation with proteins which bind fatty acids, and that inhibition of neurotransmitter amino acid uptake by unsaturated free fatty acids is reversed following the addition of proteins which possess fatty acid binding properties. Further, we have examined in detail the structural requirements for the inhibitory actions of unsaturated free fatty acids on Na^+-dependent amino acid transport by synaptosomal preparations.

Experimental Procedures

Materials. Uniformly labeled (^{14}C) amino acids and carrier-free ^{22}Na (as NaCl) were purchased from the New England Nuclear Corp. (Boston, MA) and were of the following specific activities (mCi/mmol): proline, 260; γ-aminobutyric acid, 203; glutamic acid, 254. Bovine serum albumin (fatty acid free) was obtained from Miles Laboratories (Elkhart, IN). The fatty acids and gramicidin D were purchased from Sigma Chemical Co. (St. Louis, MO). Partially purified fatty acid binding protein (M_r 12 000 fraction) was prepared from rat liver as described previously (Ockner et al., 1982). All other proteins were obtained from Calbiochem (San Diego, CA).

Transport Assays. Synaptosomal fractions were prepared from homogenates of adult Sprague-Dawley rat brain cortices by the method of Kurokawa et al. (1965). The fractions were suspended in 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. One-milliliter portions of the suspension containing 0.01–0.10 mg of synaptosomal protein were incubated with 0.1 μCi of uniformly ^{14}C-labeled amino acids (0.3–0.4 μM) under conditions described in the tables. Protein was estimated by the procedure of Lowry et al. (1951). In some experiments crude synaptosomal–mitochondrial (P2) fractions were prepared by the method of Gray & Whittaker (1962), and the uptake properties of this preparation were compared with those of the more highly purified synaptosomal fractions. Essentially the same results were obtained with both fractions. Fatty acids were added to the incubation mixtures at the required concentrations in ethanol (10 μL). At the concentration employed, ethanol had no effect on the variables studied. After incubation for various time intervals at 25 °C, reactions were terminated by the addition of 5 mL of ice-cold buffer. The synaptosomal particles were harvested on Millipore membrane filters (0.8 μm) and assayed for radioactivity as described elsewhere (Peterson & Raghupathy, 1972). We have earlier shown (Peterson & Raghupathy, 1972; Peterson et al., 1972) that under the experimental conditions employed (low synaptosomal protein concentration, short incubation period, and no added cofactors) little or no metabolism of the added ^{14}C-labeled amino acid takes place. Approximately 90% of the label was recovered in the form of the amino acid that was added to the incubation medium. In other studies, indomethacin or acetylsalicylic acid was added to the incubation medium along with fatty acids to determine whether prostaglandins or other cyclooxygenase products of fatty acids were involved in the inhibition of amino acid uptake. No effects of the prostaglandin synthesis inhibitors on the actions of free fatty acids were observed.

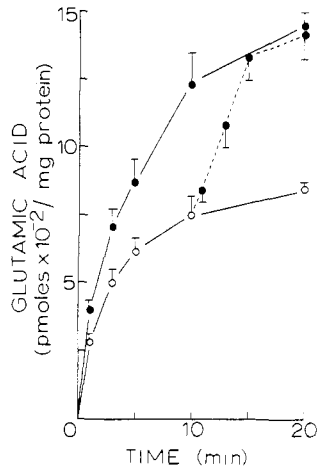

FIGURE 1: Effects of BSA on synaptosomal glutamic acid uptake. Synaptosomal amino acid accumulation was determined at various time intervals as described under Experimental Procedures. (○) Control; (●—●) BSA, 1 mg/mL, added at time zero; (●---●) BSA, 1 mg/mL, added at 10 min.

^{22}Na Flux Studies. Synaptosomal fractions (0.5 mg of protein) were incubated in 0.5 mL of a medium containing 10 mM Tris-HCl buffer, pH 7.4, 5 mM $MgCl_2$, 3 mM $CaCl_2$, 300 mM sucrose, and 1 mM KCl with 0.4 μCi of carrier-free ^{22}Na (as NaCl) and 0.5 mM ouabain for 3 min. After incubation, reactions were terminated by the addition of 5 mL of ice-cold buffer, and the synaptosomal particles were collected on Millipore filters (0.45 μm) and assayed for radioactivity by liquid scintillation spectrometry.

Analysis of Free Fatty Acids. Synaptosomal fractions were incubated under the conditions described above but in the absence of labeled amino acids. Following these incubations, the fractions were centrifuged, and lipids were extracted from the sedimented synaptosomes by the method of Folch et al. (1957). The lipid extract was subjected to thin-layer chromatography employing the solvent system petroleum ether/diethyl ether/acetic acid (90/15/1.5 v/v/v). The free fatty acids were then eluted and treated with BF_3/methanol (Supelco) 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 that was interfaced with a Hewlett-Packard 3380A digital integrator. Methyl pentadecanoate was employed as an internal standard.

Results

The synaptosomal uptake of neuroactive amino acids such as glutamic acid reached a maximum in the absence of bovine serum albumin by approximately 10 min (Figure 1). Both the initial rate of uptake and the overall level of accumulation were increased by the addition of bovine serum albumin (Figure 1). The capacity to accumulate glutamate was increased whether the albumin was added at time zero or after

[1] Abbreviations: BSA, bovine serum albumin; FABP, fatty acid binding protein; GABA, γ-aminobutyric acid; RNase, ribonuclease; Tris, tris(hydroxymethyl)aminomethane.

Table I: Effects of Proteins on Na^+-Dependent Synaptosomal Accumulation of Amino Acids

protein (1 mg/mL)	amino acid accumulation (% of control)[a] proline	GABA	glutamic acid
bovine serum albumin	149 ± 5 (19)	146 ± 7 (12)	147 ± 10 (10)
FABP (rat liver)	139 ± 5 (9)	ND[b]	ND[b]
β-lactoglobulin	138 ± 6 (12)	131 ± 6 (14)	147 ± 11 (9)
fetuin (α-fetoprotein)	133 ± 7 (6)	139 ± 8 (9)	130 ± 5 (4)
ribonuclease	97 ± 11 (6)	103 ± 8 (8)	99 ± 5 (6)
casein	92 ± 6 (6)	110 ± 8 (9)	105 ± 7 (6)
gelatin	100 ± 4 (6)	105 ± 7 (6)	102 ± 4 (4)
trypsin inhibitor	105 ± 8 (5)	99 ± 6 (9)	103 ± 8 (4)

[a] Amino acid accumulation was determined after a 10-min incubation as described under Experimental Procedures. The effects of the proteins are expressed as a percentage of the uptake occurring in their absence (control). Each value represents the mean ± SEM of the number of determinations given in parentheses. Control values for uptake were (pmol/mg of synaptosomal protein) the following: proline, 47 ± 6 (4); γ-aminobutyric acid (GABA), 352 ± 35 (4); glutamic acid, 755 ± 36 (4). [b] ND, not determined.

10 min (Figure 1). The specificity of the proteins in exerting such a stimulatory effect on amino acid uptake is demonstrated by Table I which shows that proteins with established fatty acid binding properties such as bovine serum albumin (Spector et al., 1969), hepatic fatty acid binding protein (Ockner et al., 1982), β-lactoglobulin (Spector et al., 1969), and fetuin (Parmelee et al., 1978; Carlsson et al., 1980) stimulated amino acid accumulation by 30–49% while other proteins (ribonuclease, casein, gelatin, and trypsin inhibitor) had no effect. The stimulatory effects of the various fatty acid binding proteins were concentration dependent with maximum stimulation observed at 0.5–1.0 mg/mL (data not shown).

Since the above results suggested a possible relationship between the stimulation of neuroactive amino acid transport and the sequestration of free fatty acids, we determined the free fatty acid content and composition of synaptosomal preparations incubated in the presence of BSA or ribonuclease (Table II). The principal free fatty acids associated with synaptosomes were palmitic (16:0), stearic (18:0), oleic ($18:1^{\Delta 9}$), and arachidonic ($20:4^{\Delta 5,8,11,14}$) acids. The total free fatty acid content of synaptosomal fractions increased from a zero-time value of 6.0 to 9.6 nmol/mg of protein during a 30-min incubation period. Increases were observed in the levels of the individual free fatty acids as well. Such increases presumably reflect lipolysis of fatty acid esters, e.g., phospholipids. When the synaptosomal fractions were incubated in the presence of BSA (1 mg/mL), the total free fatty acid content was reduced to 3.0 nmol/mg of protein. Similar decreases were observed in the levels of palmitic, stearic, oleic, and arachidonic acids. In contrast, when synaptosomal fractions were incubated in the presence of RNase for 30 min, the fatty acid levels continued to increase, and the total fatty acid content at the end of 30 min was 10.6 nmol/mg of protein. The levels of individual fatty acids in the preparations incubated in the presence of ribonuclease were no different than those obtained in control incubations (without protein).

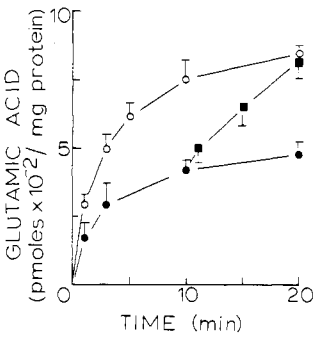

FIGURE 2: Inhibition of synaptosomal glutamic acid uptake by arachidonic acid and its reversal by BSA. Synaptosomal amino acid accumulation was determined at various time intervals as described under Experimental Procedures. (○) Control; (●) arachidonic acid, 5 μM; (■) arachidonic acid, 5 μM, plus BSA, 1 mg/mL.

The addition of oleic acid to the incubation medium employed for amino acid uptake studies resulted in a dose-dependent inhibition of sodium-dependent (amino acid) accumulation (Rhoads et al., 1982d). A similar dose response for inhibition of sodium-dependent amino acid uptake was obtained with the other endogenous unsaturated fatty acid, arachidonic acid (data not shown). This inhibition was the result of both a decreased initial rate of transport and a reduced capacity to accumulate amino acids (Figure 2). The data presented in Table III demonstrate that the inhibition of amino acid uptake by the unsaturated fatty acids, oleate and arachidonate, was reversed by proteins that have the ability to bind fatty acids and not by ribonuclease. The observation that partially purified FABP was somewhat less effective in this regard than BSA may reflect the fact that, whereas BSA is fatty acid free, FABP contains significant amounts of bound, endogenous free fatty acids (Ockner et al., 1982). An important aspect of this reversibility is the ability of the fatty acid binding proteins to return the uptake to control levels when added after the uptake had reached an apparent maximum in the presence of an inhibitory fatty acid (Figure 2).

As described previously (Rhoads et al., 1982d), the saturated fatty acids, palmitate and stearate, had negligible effects on proline transport. Additional experiments were performed with glutamic acid uptake to determine the structural requirements for fatty acid inhibition at a single fatty acid concentration (10 μM), and the results are summarized in Table IV. All of the cis-unsaturated fatty acids below 22 carbons in chain length substantially diminished glutamate uptake to levels which were 17–39% of the control levels. The 22-carbon, polyunsaturated docosahexaenoic ($22:6^{\Delta 4,7,10,13,16,19}$) acid had inhibitory effects similar to the shorter chain unsaturated fatty acids. The trans-monounsaturated fatty acids examined (*trans*-$18:1^{\Delta 9}$, *trans*-$18:1^{\Delta 11}$) were much less effective inhibitors than their cis-unsaturated analogues. The presence of a hy-

Table II: Effects of BSA and Ribonuclease on the Free Fatty Acid Content of Synaptosomal Fractions

addition	time of incubation (min)	free fatty acid content (nmol/mg of protein)[a] palmitic acid (16:0)	stearic acid (18:0)	oleic acid ($18:1^{\Delta 9}$)	arachidonic acid ($20:4^{\Delta 5,8,11,14}$)	total
none	0	1.5 ± 0.2	2.0 ± 0.3	1.0 ± 0.1	1.5 ± 0.2	5.9 ± 0.7
none	30	2.5 ± 0.1	3.0 ± 0.2	2.0 ± 0.3	2.1 ± 0.2	9.6 ± 0.7
BSA (1 mg/mL)	30	1.1 ± 0.4	1.2 ± 0.5	0.6 ± 0.2	0.2 ± 0.1	3.0 ± 1.0
ribonuclease (1 mg/mL)	30	2.6 ± 0.3	3.2 ± 0.4	2.4 ± 0.8	2.4 ± 0.4	10.6 ± 1.8

[a] Means ± SEM for four independent determinations. Each determination was obtained from 10–20 mg of synaptosomes incubated in the presence or absence of the indicated protein.

Table III: Effects of Bovine Serum Albumin, Ribonuclease, and Rat Liver FABP on the Inhibition of Synaptosomal Amino Acid Uptake by Unsaturated Free Fatty Acids

addition	amino acid accumulation[a] proline pmol/mg	proline % of control	GABA pmol/mg	GABA % of control
none	47 ± 6	100	353 ± 35	100
oleic acid (25 μM)	21 ± 2	45	175 ± 30	50
arachidonic acid (10 μM)	11 ± 4	23	ND[b]	ND[b]
oleic acid + BSA (1 mg/mL)	55 ± 5	117	466 ± 46	132
oleic acid + ribonuclease (1 mg/mL)	22 ± 2	47	189 ± 38	54
oleic acid + rat liver FABP (1 mg/mL)	38 ± 4	81	ND[b]	ND[b]
arachidonic acid + BSA (1 mg/mL)	48 ± 7	102	ND[b]	ND[b]

[a] Amino acid accumulation was determined after a 10-min incubation as described under Experimental Procedures. Fatty acids were added at the concentrations indicated in ethanol. [b] ND, not determined.

Table IV: Comparison of the Effects of Different Fatty Acids on Synaptosomal Glutamic Acid Uptake

fatty acid[a]	uptake of glutamic acid[b] pmol/mg	% of control
none (control)	758 ± 62 (6)	100
$18:1^{\Delta 6}$	128 ± 19 (3)	17
$18:1^{\Delta 9}$	139 ± 19 (5)	18
$18:1^{\Delta 11}$	162 ± 13 (4)	21
$20:4^{\Delta 5,8,11,14}$	190 ± 101 (5)	25
$22:6^{\Delta 4,7,10,13,16,19}$	229 ± 44 (5)	30
$18:2^{\Delta 9,12}$	243 ± 77 (4)	32
$18:3^{\Delta 9,12,15}$	265 ± 12 (4)	35
$16:1^{\Delta 9}$	273 ± 35 (4)	36
$20:1^{\Delta 11}$	296 ± 28 (4)	39
$18:1^{\Delta 9}$-OH	409 ± 34 (4)	54
$18:1^{\Delta 9}$-OCH_3	478 ± 31 (4)	63
trans-$18:1^{\Delta 9}$	486 ± 16 (3)	64
trans-$18:1^{\Delta 11}$	512 ± 46 (4)	67
$22:1^{\Delta 13}$	633 ± 60 (4)	84
$24:1^{\Delta 15}$	663 ± 53 (4)	87
12-OH, $18:1^{\Delta 9}$	723 ± 83 (3)	95
8:0	847 ± 47 (3)	112
12:0	728 ± 31 (3)	96
16:0	796 ± 15 (3)	105
18:0	807 ± 27 (3)	106

[a] Fatty acids were added in ethanol to a final concentration of 10 μM (equivalent to 1 nmol/μg of synaptosomal protein). 12-OH, $18:1^{\Delta 9}$ is 12-hydroxyoleic acid, $18:1^{\Delta 9}$-OH is oleoyl alcohol, and $18:1^{\Delta 9}$-OCH_3 is oleic acid methyl ester. [b] The accumulation of glutamate was determined following a 10-min incubation as described under Experimental Procedures. Values are expressed both as the mean ± SEM of the number of determinations in parentheses and as a percentage of the control uptake (no added fatty acids).

droxyl group (12-OH, $18:1^{\Delta 9}$) in the oleic acid molecule ($18:1^{\Delta 9}$) resulted in total loss of the inhibitory capacity. The inhibitory capacity of oleic acid was also reduced upon modification of the polar (carboxyl) group since both oleoyl alcohol (18:1-OH) and oleic acid methyl ester (18:1-OCH_3) showed a reduced capacity for inhibition compared to the free acid. Similar results were obtained with arachidonic acid, arachidonyl alcohol, and arachidonate methyl ester (data not shown). Saturated fatty acids from 8 to 18 carbons in chain length had no significant effects on glutamate transport.

The possibility that oleic acid alters Na^+ gradients by a direct ionophoretic action was investigated by $^{22}Na^+$-flux studies. As can be seen from the data presented in Table V, gramicidin D at a concentration of 6 μg/mL significantly increased the influx of $^{22}Na^+$ into synaptosomal fractions while oleic acid (50 μM) had no effect.

Table V: Effects of Gramicidin D and Oleic Acid on $^{22}Na^+$ Influx into Synaptosomes[a]

addition	$^{22}Na^+$ uptake (cpm)
none	5656 ± 446
gramicidin D (6 μg/mL)	20974 ± 1415
oleic acid (50 μM)	5725 ± 273

[a] Synaptosomes were incubated for 3 min with 0.4 μCi of $^{22}Na^+$ under conditions described in the text. The values for ^{22}Na uptake are the means ± SEM of four independent determinations, each performed in duplicate.

Discussion

Although the amino acid uptake under the experimental conditions described in this paper is referred to as synaptosomal uptake, the possibility that other elements in the synaptosomal preparation (such as glial cell fragments) also contribute to the uptake cannot be overlooked. However, the amino acids described in this paper are all taken up by high-affinity, Na^+-dependent processes. These transport systems are sensitive to inhibition by veratridine (Rhoads et al., 1982b) and *Tityus serrulatus* venom (Rhoads et al., 1982c,e), and such inhibition can be reversed by tetrodotoxin. These observations strongly suggest that the uptake is mediated by presynaptic terminals with functionally intact Na^+ channels.

The results presented in this paper extend our earlier observations on the effects of serum albumin and of free fatty acids on synaptosomal uptake of neurotransmitter amino acids and demonstrate a possible regulatory role for endogenous free fatty acids in membrane function. The stimulation of Na^+-dependent amino acid uptake is observed with a number of proteins that are known to bind free fatty acids. In contrast, proteins that do not possess fatty acid binding properties do not stimulate Na^+-dependent amino acid uptake. These data suggest that proteins that stimulate synaptosomal amino acid uptake do so by binding endogenous free fatty acids which otherwise inhibit uptake. Additional support for this conclusion was obtained from our data on the free fatty acid composition of synaptosomal fractions incubated in the presence of bovine serum albumin or ribonuclease. In the absence of exogenously added fatty acid binding proteins or in the presence of proteins which do not bind fatty acids, the amounts of palmitic, stearic, oleic, and arachidonic acids associated with synaptosomal preparations increased during a 30-min incubation at 25 °C. Conversely, when the fractions were incubated with bovine serum albumin, the free fatty acid levels were significantly less than the control values. These data imply that the stimulatory effect of proteins on synaptosomal amino acid uptake is related to their capacity to reduce the free fatty acid content of the synaptosomal membranes. A corollary to this conclusion is that endogenous free fatty acids modulate the Na^+-dependent amino acid transport activity of synaptosomal fractions.

Several membrane-associated cellular processes have been shown to be altered by free fatty acids. Among these are amino acid transport in membrane vesicles prepared from *Escherichia coli* (Goto & Mizushima, 1978), surface receptor capping in lymphocytes (Klausner et al., 1980a), mitochondrial coupling of respiration and oxidative phosphorylation (Heaton & Nicholls, 1976), and membrane-bound enzyme activity (Ahmed & Thomas, 1971). Free fatty acids alter synaptosomal amino acid uptake into both intact synaptosomes

(Rhoads et al., 1982d) and plasma membrane vesicles derived from synaptosomes (Rhoads et al., 1982c). These alterations were specific in two respects. First, only sodium-dependent transport was inhibited; sodium-independent transport systems were unaffected. Second, saturated fatty acids had little or no effect. Such specificity indicates, along with other aspects of the inhibition such as its reversibility by fatty acid binding proteins and the relatively low concentrations of fatty acids needed for inhibition, that the inhibition is not associated with a general detergent action but with some more specific mechanism of inhibition such as alteration of a particular membrane lipid domain (see below). In this paper we have demonstrated the presence of free fatty acids in synaptosomes; comparison of the endogenous free fatty acids present in synaptosomes with the effects of exogenous fatty acids, suggests that oleic and arachidonic acids are most likely to act as inhibitors in vivo.

Certain basic conclusions can be drawn from the correlation between the structure of the fatty acid molecule and the extent of inhibition of Na^+-dependent amino acid transport. At least one double bond is required to effect substantial inhibition; thus, palmitic, stearic, lauric (12:0), and caprylic (8:0) acids, which lack double bonds, have little or no effect. Among the monounsaturated fatty acids of chain length less than 22 carbons, the position of the double bond in the molecule apparently has little influence, whereas the longer chain ($\geq$22 C) monounsaturated fatty acids have little or no effect on glutamic acid uptake. The configuration of the double bond also has a significant effect on the inhibition. Fatty acids with cis configurations (i.e., those with a "bend" in the acyl chain) are far more inhibitory than those with the trans configuration. Finally, oleic acid was more inhibitory than its corresponding alcohol or methyl ester, suggesting that the charge at the polar end of the fatty acid molecule also plays a role in the inhibitory action.

Several lines of evidence suggest that the inhibition of neuroactive amino acid transport by unsaturated fatty acids is not due to disturbances in Na^+-ion gradients. The observation that the inhibition of proline, glutamic acid, and GABA uptake was not reversed by tetrodotoxin (data not shown) suggests that the inhibition is not the result of depolarization of synaptosomes mediated by voltage-sensitive Na^+ channels. Furthermore, fatty acid inhibition was observed in both synaptosomes where the transport is driven via (Na^+,K^+)-ATPase and synaptosomal vesicles where the uptake is driven solely by an artificial Na^+ gradient (Rhoads et al., 1982c). This rules out inhibition of (Na^+,K^+)-ATPase (Ahmed & Thomas, 1971) as a primary mechanism of action. Finally, oleic acid does not alter the influx of $^{22}Na^+$ in synaptosomal preparations (Table V).

The differential effects of free fatty acids presented in this paper are consistent with the model proposed by Klausner et al. (1980b) for the interaction of free fatty acids with cell membranes and lipid bilayers. Free fatty acids readily intercalate into the plasma membranes of cells and remain there in unesterified form for a short period of time. From their studies, Klausner et al. (1980b) have shown that fatty acids can be divided into two primary groups on the basis of their effects on lipid domains. According to this model, cis-unsaturated fatty acids (group A) preferentially partition into fluid phase of the membranes while saturated and trans-unsaturated fatty acids (group B) partition into the gel phase. Their data support the view that there are discrete lipid domains in the membrane and that several biological effects of free fatty acids, which differ between saturated and unsaturated acids, may be explained by the preferential partitioning of these acids in the membrane. Our results may be interpreted by this model. While there is a wide range of inhibition seen in our study, the differential effects of free fatty acids fall into two general categories. One contains all tested cis-unsaturated fatty acids except for the monounsaturates with more than 20-carbon atoms. The second group consists of the longer chain monounsaturated acids $22{:}1^{\Delta 13}$ and $24{:}1^{\Delta 15}$, which have melting points comparable to those of saturated fatty acids, as well as saturated, trans-unsaturated, and hydroxy fatty acids. These differential effects also extend to the type of transport affected by free fatty acids, since we have shown previously that only the Na^+-dependent systems involving putatative neurotransmitter amino acids are inhibited (Rhoads et al., 1982d). On the basis of these considerations, we may conclude that Na^+-dependent neurotransmitter amino acid transport carrier proteins reside in a relatively fluid domain in the synaptosomal membrane and that unsaturated free fatty acids exert a regulatory effect by interactions with such domains. Fatty acid binding proteins, endogenous or exogenous, may play an important role in the transport process by virtue of their effects on these domains, consequent to the removal from them of free fatty acids. The recent studies of Gould et al. (1982) on lipid effects on the binding properties of a reconstituted insulin receptor are particularly relevant. These authors have shown that the insulin receptor had a greater affinity for insulin when it was reconstituted in a saturated lipid environment than in an unsaturated environment. They suggest that membrane lipid environment, especially the degree of unsaturation of the phospholipid acyl chains, can influence the binding properties of the receptor protein. Our data are fully in accord with the hypothesis that the nature of the surrounding lipid environment can markedly influence the functional components residing within the membrane.

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Registry No. $18{:}1^{\Delta 6}$, 593-39-5; $18{:}1^{\Delta 9}$, 112-80-1; $18{:}1^{\Delta 11}$, 506-17-2; $20{:}4^{\Delta 5,8,11,14}$, 506-32-1; $22{:}6^{\Delta 4,7,10,13,16,19}$, 6217-54-5; $18{:}2^{\Delta 9,12}$, 60-33-3; $18{:}3^{\Delta 9,12,15}$, 463-40-1; $16{:}1^{\Delta 9}$, 373-49-9; $20{:}1^{\Delta 11}$, 5561-99-9; $18{:}1^{\Delta 9}$-OH, 143-28-2; $18{:}1^{\Delta 9}$-OCH_3, 112-62-9; *trans*-$18{:}1^{\Delta 9}$, 112-79-8; *trans*-$18{:}1^{\Delta 11}$, 693-72-1; 16:0, 57-10-3; 18:0, 57-11-4; GABA, 56-12-2; proline, 147-85-3; glutamic acid, 56-86-0.

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Lipid Requirements for Coupled Cytochrome Oxidase Vesicles†

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ABSTRACT: Cytochrome *c* oxidase has been reconstituted with two synthetic phospholipids, dioleoylphosphatidylcholine and dioleoylphosphatidylethanolamine. Vesicles prepared from either of these two lipids alone showed no stimulation of enzyme activity upon addition of carbonyl cyanide (trifluoromethoxy)phenylhydrazone and valinomycin, indicating that they were leaky to small ions. However, when mixtures of the two lipids were used for the reconstitution, tightly coupled vesicles could be obtained. The coupling ratio was dependent upon the ratio of dioleoylphosphatidylcholine to dioleoylphosphatidylethanolamine and also on the lipid-to-protein ratio. Maximal rates of enzyme activity were not significantly different with different lipid mixtures. The results are discussed in terms of both the size distribution of the reconstituted vesicles and the possible requirement for a variety of lipid species to ensure tight sealing at the lipid–protein interface.

Cytochrome *c* oxidase is a multisubunit enzyme spanning the inner mitochondrial membrane (Hackenbrook & Hammon, 1975) and functions as the terminal component in the electron transport chain. It can be incorporated into vesicles of a defined lipid composition, and a number of workers have studied the lipid requirements for enzyme activity. Vik & Capaldi (1977) have demonstrated that the enzyme functions optimally when reconstituted with phospholipids containing long unsaturated fatty acyl chains. Other than this requirement for a fluid matrix, no head group specificity has been observed apart from a possible requirement for tightly bound cardiolipin (Robinson et al., 1980).

Reconstituted vesicles exhibiting respiratory control can be prepared, and these show maximal rates of enzyme activity only in the presence of uncoupling agents such as carbonyl cyanide (trifluoromethoxy)phenylhydrazone (FCCP) plus valinomycin (Hinkle et al., 1972). High levels of respiratory control are obtained when cytochrome *c* oxidase is reconstituted with partially purified soya bean phospholipid (Hinkle et al., 1972), with phospholipid extracted from ox heart mitochondria (Hunter & Capaldi, 1974), or with mixtures of purified phospholipids (Racker, 1973). When the enzyme is incorporated into vesicles composed of a single lipid species, generally low levels of respiratory control are observed. In addition, it has been observed that tightly sealed vesicles are produced only when the enzyme is reconstituted in the presence of a large excess of lipid (Vik & Capaldi, 1977). We have characterized the lipid requirements for coupling by reconstituting the oxidase in either dioleoylphosphatidylcholine, dioleoylphosphatidylethanolamine, or mixtures of these two synthetic phospholipids at various lipid-to-protein ratios.

Materials and Methods

Cytochrome *c* oxidase was prepared by the method of Kuboyama et al. (1972) and stored in liquid nitrogen. Dioleoylphosphatidylcholine (DOPC) and dioleoylphosphatidylethanolamine (DOPE) were synthesized as described previously (Cullis & De Kruijff, 1976) and were at least 99% pure as determined by thin-layer chromatography.

Cytochrome *c* (horse heart, type VI), sodium cholate, carbonyl cyanide (trifluoromethoxy)phenylhydrazone (FCCP),

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