Arachidonic acid inhibits glycine transport in cultured glial cells

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The effects of arachidonic acid on glycine uptake, exchange and efflux in C6 glioma cells were investigated. Arachidonic acid produced a dose-dependent inhibition of high-affinity glycine uptake. This effect was not due to a simple detergent-like action on membranes, as the inhibition of glycine transport was most pronounced with *cis*-unsaturated long-chain fatty acids, whereas saturated and *trans*-unsaturated fatty acids had relatively little or no effect. Endogenous unsaturated non-esterified fatty acids may exert a similar inhibitory effect on the transport of glycine. The mechanism for this inhibitory effect has been examined in a plasma membrane vesicle preparation derived from C6 cells, which avoids metabolic or compartmentation interferences. The results suggest that part of the selective inhibition of glycine transport by arachidonic acid could be due to the effects of the arachidonic acid on the lipid domain surrounding the carrier.

INTRODUCTION

Glycine, besides its numerous metabolic functions (Meister, 1975), is widely held to fulfil most of the criteria for an inhibitory neurotransmitter in the mammalian nervous system, mainly in the spinal cord (Aprison & Daly, 1978) and probably in some localized areas of the brain such as the substantia nigra (Pycocock *et al.*, 1981).

Several lines of evidence suggest that the glial cells of the central nervous system play an important role in the modulation of neuronal excitability via control of the levels of neuroactive substances in the extracellular milieu of neurons (Schrier & Thompson, 1974; Henn, 1976; Schousboe *et al.*, 1977). The re-uptake of neurotransmitter substances into presynaptic nerve endings or glial cells provides one way of clearing the extracellular space of potential neuroactive substances, and so constitutes an efficient mechanism by which postsynaptic action can be terminated. Specific high-affinity transport systems have been identified in glia for several putative amino acid neurotransmitters including γ -aminobutyric acid (GABA), taurine, glycine, glutamic and aspartic acid (Henn & Hamberger, 1971; Hertz *et al.*, 1978; Waniewski & Martin, 1984; Holopainen & Kontro, 1986; Zafra & Giménez, 1986a). Moreover, recent results from our laboratory have demonstrated the existence of a heterogeneous system of transport for glycine in glioma cells on the basis of ionic dependency and kinetic features (Zafra & Giménez, 1989a). One system is Na^+ - and Cl^- -dependent with high affinity for the substrate, and this corresponds to the characteristics of System Gly [specific for this amino acid and its *N*-methylated derivative sarcosine (Christensen & Handlogten, 1981)]. The low-affinity component of the transport of glycine can be divided into two components, namely System A (effective for amino acids having short, polar or linear side chains) and System ASC (able to transport alanine, serine and cysteine), both Na^+ -dependent but Cl^- -independent. System Gly showed kinetic constants and energetic requirements similar to those found in nerve terminals (Mayor *et al.*, 1981).

Numerous studies have shown that non-esterified fatty acids, which are present in the brain in very small concentrations under normal conditions, rapidly accumulate during cerebral ischaemia or hypoxia and in a number of other conditions such as

hypoglycaemia, drug-induced seizures and electroconvulsive shock (Bazan, 1970, 1971; Avelandano & Bazan, 1975; Broniszewska-Ardelt & Dabrowiecki, 1976; Gardier *et al.*, 1981; Siesjö *et al.*, 1982). Unsaturated fatty acids inhibit the Na^+ -dependent uptake of several amino acid neurotransmitters, including glutamate, aspartate and GABA (Rhoads *et al.*, 1982; Chan *et al.*, 1983; Troeger *et al.*, 1984; Peterson & Raghupathy, 1985; Barbour *et al.*, 1989). This inhibition is specific for Na^+ -dependent uptake processes and for unsaturated fatty acids (Rhoads *et al.*, 1982, 1983a).

The results in the present paper demonstrate that arachidonic acid inhibits high-affinity glycine uptake into the glial cells by a mechanism that is independent of the source of energy that drives the transport, and suggest that unsaturated fatty acids alter the lipid environment surrounding the carrier protein and hence modify its function.

EXPERIMENTAL

Materials

[U- ^{14}C]Glycine (113 mCi/mmol) was obtained from Amersham International, [$2\text{-}^3\text{H}$]glycine (49.0 Ci/mmol) and carrier-free $^{22}\text{Na}^+$ (as NaCl) (110 mCi/mg) were purchased from New England Nuclear, Boston, MA, U.S.A. Dulbecco's modified Eagle's medium (DMEM), Earle's salts and fetal calf serum were obtained from Gibco, Paisley, Scotland, U.K. Dishes for tissue culture were purchased from Costar, Cambridge, MA, U.S.A., and valinomycin, phospholipase A_2 , BSA, non-esterified fatty acids and derivatives were purchased from Sigma. The lipophilic cation [^3H]tetraphenylphosphonium bromide (2.5 Ci/mmol) was a gift from Dr. H. R. Kaback (Roche Institute for Molecular Biology, Nutley, NJ, U.S.A.). Ficoll was provided by Pharmacia, and was exhaustively dialysed against water before use. All other reagents used were of the highest purity available. Non-esterified fatty acids and derivatives were maintained in ethanol at -70°C under an atmosphere of N_2 in order to minimize further oxidation.

Cell culture

The cell line were obtained from the American Type Culture Collection. Cultures were grown at 37°C in a humidified

Abbreviations used: GABA, γ -aminobutyric acid; DMEM, Dulbecco's modified Eagle's medium; $\Delta\Psi$, potential across the plasma membrane; PBS, phosphate-buffered saline.

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atmosphere of CO₂/air (1:19). The growth medium was changed at 3-day intervals, and the medium contained 10% fetal calf serum. Only cell cultures at the stationary growth phase were used for cell membrane isolation.

Membrane vesicle preparation

Membrane vesicles were prepared essentially as described previously (Zafra & Giménez, 1986a). Cells from about 20 confluent roller bottles were used for each membrane preparation. Bottles were rinsed twice with 0.3 M-mannitol, 1 mM-EDTA, 10 mM-Tris/HCl, pH 7.5 (solution 1), and then cells were harvested by scraping with a rubber blade. Subsequent steps were carried out at 4 °C. Cells were centrifuged at 3000 *g* for 10 min, resuspended in 60 ml of solution 1 and homogenized with 10 strokes of an all-glass Potter-Elvehjem homogenizer with a tight-fitting pestle. The homogenate was centrifuged for 10 min at 3000 *g* in a Sorvall SS34 rotor and the pellet was washed by resuspension in 30 ml of solution 1 and centrifugation at 3000 *g* for 10 min. Both supernatants were combined (S1) and centrifuged for 20 min at 27000 *g* to obtain a crude mitochondrial pellet (P₂). P₂ was resuspended in about 3 ml of solution 1 and layered over two discontinuous gradients, each consisting of five layers of 20%, 16%, 12%, 8% and 2% Ficoll in solution 1. The gradients were then centrifuged in a Beckman SW28 rotor at 75000 *g* for 90 min. The 2–8% and 8–12% interfaces (F₁ and F₂ respectively) were collected, combined and diluted with 4 vol. of solution 1. F1 and F2 were centrifuged at 27000 *g* for 20 min. The pellet was subjected to osmotic disruption in 20 ml of 5 mM-Tris/HCl/1 mM-EDTA (pH 7.4). After stirring for 45 min the suspension was centrifuged again (20 min at 27000 *g*) and the pellet was resuspended in 10 ml of a 300 mosm medium, pH 7.4, with the ionic composition of the medium depending on the particular experiment. Finally, the suspension was centrifuged in the 300 mosm medium to a protein concentration of 5 mg/ml. Portions were frozen in liquid N₂ and stored at –70 °C. Under these conditions, membrane vesicles were functional for at least 1 month.

Transport assays

In C6 cells. All solutions used in the uptake experiments were prepared with distilled deionized water and filtered through Millipore filters (0.45 µm pore size) to avoid possible bacterial contamination. The osmolarity of all solutions was kept constant during the uptake experiments. The growth medium was removed by aspiration and, after washing in Earle's medium (37 °C), cells were harvested by scraping with a rubber blade and suspended in Earle's medium. The viability of these dissociated cells was measured by Trypan Blue exclusion. About 90% of these cells were viable. The uptake was started by adding 100 µl of Earle's medium containing radioactive glycine (0.1 µCi) and the indicated fatty acids to 20 µl portions of C6 cells (about 60 µg of protein). Cells were incubated for the indicated time and then the experiments were terminated by dilution with 2 ml of ice-cold phosphate-buffered saline (PBS; 0.9 mM-CaCl₂, 2.7 mM-KCl, 1.5 mM-KH₂PO₄, 0.5 mM-MgCl₂, 136.9 mM-NaCl, 7.37 mM-Na₂HPO₄, pH 7.4) and immediate filtration through a moistened Millipore filter HAWP (0.45 µm pore size) attached to a vacuum assembly. The filters were washed once with ice-cold PBS. The dilution, filtration and washing procedures were performed within 15 s. The filters were dried at 60 °C, placed in microvials and their radioactivity was measured in a liquid scintillation counter (LKB 1219 Rackbeta). Results were corrected for background radioactivity by using a control before adding the radioactive substrate solution. All incubations were carried out in triplicate. Each experiment was repeated at least three times with different cell cultures. For estimating statistical differences,

the data were compared using Student's *t* test; differences at the 0.05 level were considered to be statistically significant.

In plasma membrane vesicles from C6 cells. Aliquots of 20 µl of the suspension of membrane vesicles (about 100 µg of protein) were preincubated for the indicated times at 25 °C in the absence or presence of fatty acids. The uptake was started by adding 100 µl of a solution containing [U-¹⁴C]glycine (10 µM final concentration) in 150 mM-NaCl, 1 mM-MgSO₄, 5 mM-Hepes/Tris, pH 7.4 (NaCl medium), or 150 mM-KCl, 1 mM-MgSO₄, 5 mM-Hepes/Tris, pH 7.4 (KCl medium). The experiments were terminated by diluting with 5 ml of ice-cold 0.8 M-NaCl and immediately filtering through a moistened Millipore filter HAWP (0.45 µm pore size) attached to a vacuum assembly. The filters were processed as indicated above. When ionophore was used, it was preincubated with the membrane suspension for 1 min.

Efflux from actively loaded vesicles

Portions of 10 µl (about 45 µg of protein) were preincubated for 2 min at 25 °C. Uptake was carried out for 2 min with 30 µM-[¹⁴C]glycine as indicated in the influx experiments. After the indicated time, the incubation mixtures were diluted 20-fold with the NaCl medium containing (or not) fatty acids. The experiment was terminated by dilution and filtration as indicated above.

Protein determination

Membrane proteins were determined according to the method of Resch *et al.* (1971).

Analysis of the data

Calculation of activation energies (*E_a*) was according to the Arrhenius equation, which may be written as:

$$\log K = -\frac{E_a}{2.3RT} \cdot \frac{1}{T}$$

where *K* is the rate constant, *R* is the gas constant and *T* is the absolute temperature. In practice, the numerical value of *E_a* is determined from the slope of a plot of log *K* versus 1/*T*.

RESULTS

The effect of arachidonic acid on high-affinity glycine uptake was tested in C6 cells incubated with [¹⁴C]glycine at a concentration (10 µM) which serves as substrate for the high-affinity glycine transport system with little involvement of the low-affinity component (Zafra & Giménez, 1989a). Arachidonic acid inhibited glycine transport, with a tendency to produce a greater inhibition with increasing incubation time. Fig. 1 shows the effects of a number of fatty acids at different concentrations on glycine uptake. Arachidonic acid exerted a concentration-dependent inhibition of glycine uptake, with 50% inhibition at 0.2 nmol of arachidonic acid/µg of protein. However, saturated fatty acids such as myristic, palmitic or stearic acid had no appreciable effect on glycine uptake.

The structural requirements for inhibition by a fatty acid at a single concentration (0.30 nmol/µg of protein) are presented in Table 1. All *cis*-unsaturated fatty acids tested decreased the uptake of glycine. The extent of inhibition (docosahexanoic ≈ arachidonic > linolenic ≈ linoleic > erucic ≈ oleic) can be correlated with the number of double bonds but not with the length of the chain (C_{18:1} ≈ C_{22:1}). The *trans*-unsaturated fatty acids examined (elaidic acid and linolelaidic acid) were much less effective inhibitors than their *cis*-unsaturated analogues oleic acid and linoleic acid respectively. The inhibitory capacity of

Fig. 1. Effect of the concentration of fatty acids on the uptake of glycine in glial cells

C6 glioblastoma cells were incubated for 45 min at 37 °C in Earle's medium in the presence of the indicated concentrations of palmitic acid (▲), myristic acid (■) or arachidonic acid (●) and 10 μ M-[14 C]glycine. Values represent % of control values in the absence of arachidonic acid; 100 % corresponds to 517 ± 29 pmol/mg of protein.

Fig. 2. Effect of arachidonic acid on the uptake of glycine into plasma membrane vesicles

Plasma membrane vesicles from C6 glioblastoma cells were preincubated for 15 min at 25 °C in the presence of the indicated concentrations of arachidonic acid. The transport assay was carried out for 15 min at 25 °C in the presence of 10 μ M-[14 C]glycine and an artificial NaCl gradient (out > in); 100 % corresponds to 219 ± 3 pmol/min per mg of protein.

arachidonic acid was also decreased upon modification of the polar (carboxyl) group, since both arachidonyl alcohol and arachidonic methyl ester showed a decreased capacity for inhibition compared with the free acid (Table 1).

Previous work has shown that the energy which drives the uptake of glycine is derived from both the Na⁺ gradient created by the (Na⁺-K⁺)ATPase and the electrical potential (Zafra & Giménez, 1989a,b). Fig. 2 shows that arachidonic acid inhibits the transport of glycine into plasma membrane vesicles, where the uptake is driven solely by artificial Na⁺ and Cl⁻ gradients.

Table 1. Effect of chain length and the degree of saturation on the high-affinity uptake of glycine

C6 glioblastoma cells were incubated for 45 min at 37 °C in Earle's medium in the presence of the indicated fatty acids and derivatives (0.30 nmol/μg of protein) and 10 μ M-[14 C]glycine. Values are the means $\pm$ S.E.M. of three replicate determinations. Values in parentheses represent % of control values in the absence of fatty acids. Values were compared with control values by using Student's *t* test: ** *P* < 0.01; *** *P* < 0.001.

Addition	High-affinity uptake (pmol/45 min per mg of protein)
None	510 $\pm$ 30 (100)
Oleic acid (C _{18:1,Δ9})	326 $\pm$ 23** (64)
Elaidic acid (<i>trans</i> -C _{18:1,Δ9})	470 $\pm$ 15 (92)
Linoleic acid (C _{18:2,Δ9,12})	129 $\pm$ 14*** (25)
Linolelaidic acid (<i>trans</i> -C _{18:2,Δ9,12})	270 $\pm$ 19** (53)
Linolenic acid (C _{18:3,Δ9,12,15})	122 $\pm$ 10*** (24)
Arachidonic acid (C _{20:4,Δ5,8,11,14})	46 $\pm$ 20*** (9)
Erucic acid (C _{22:1,Δ13})	316 $\pm$ 10** (62)
Docosahexaenoic acid (C _{22:6,Δ4,7,10,13,16,19})	31 $\pm$ 08*** (6)
Arachidonyl alcohol	256 $\pm$ 14** (50)
Arachidonyl methyl ester	270 $\pm$ 17** (52)

The effect of arachidonic acid on glycine transport under equilibrium conditions is shown in Table 2. The uptake of labelled glycine into vesicles which had been preloaded with unlabelled glycine was significantly inhibited in the presence of arachidonic acid.

Table 3 shows that BSA, a protein with established fatty acid-binding properties, can reverse the inhibitory effect of arachidonic acid. On the other hand, the uptake of leucine, which proceeds via an Na⁺-independent mechanism, remained unaffected in the presence of arachidonic acid concentrations that inhibit the transport of glycine (results not shown).

The possibility that arachidonic acid alters Na⁺ gradients by a direct ionophoretic action was investigated by 22 Na⁺-influx studies (Fig. 3). No significant changes were found in the presence of 0.25 nmol of arachidonic acid/μg of protein.

The relationship between the electrical potential and the inhibitory effect of arachidonic acid is shown in Table 4. Valinomycin, an ionophore that specifically increases the mem-

Table 2. Effect of arachidonic acid on glycine exchange

Plasma membrane vesicles from C6 glioma cells preloaded with NaCl medium and 100 μ M-glycine were preincubated for 15 min at 25 °C in the same medium and in the presence or absence of arachidonic acid. Glycine uptake due to exchange was carried out for 10 min in the presence of 0.2 μ M-[3 H]glycine. Values are the means $\pm$ S.E.M. of three replicate determinations. Values in parentheses represent % of control values in the absence of fatty acid. Values were compared with control values by using Student's *t* test: ** *P* < 0.01; *** *P* < 0.001.

Arachidonic acid (nmol/μg of protein)	[3 H]Glycine uptake due to exchange (pmol/10 min per mg of protein)
0	54.61 $\pm$ 0.41 (100)
0.50	17.24 $\pm$ 0.08** (32)
0.75	9.69 $\pm$ 0.02*** (18)

Table 3. Reversibility of the effect of fatty acids on glycine uptake

Plasma membrane vesicles from C6 glioblastoma cells preloaded with KCl medium were preincubated for 15 min at 25 °C in the absence or presence of arachidonic acid. Afterwards BSA was added and the incubation was continued for 15 min. Valinomycin was added 1 min before the transport assay was started. The transport assay was carried out for 15 min in the presence of 10 μM - ^{14}C glycine and an artificial Na^+ gradient (out > in). Values are the means $\pm$ S.E.M. of three replicate experiments. Values in parentheses represent % of control values. Values were compared with control values by using Student's *t* test: ** *P* < 0.01.

Addition	High-affinity glycine uptake (pmol/15 min per mg of protein)
None	228 $\pm$ 3 (100)
BSA (5 mg/ml)	208 $\pm$ 7 (91)
Arachidonic acid (0.25 nmol/ μg of protein)	110 $\pm$ 2** (48)
Arachidonic acid + BSA	203 $\pm$ 3 (89)

brane permeability to K^+ , producing a transient increase in the membrane potential (negative inside) (Scott & Nicholls, 1980), stimulated the uptake of glycine (20 % over control). When both valinomycin and arachidonic acid were present during the incubation, there was a slight increase in glycine uptake compared with the level obtained in the sole presence of arachidonic acid (8 %); however, this uptake was still much lower than in the controls (in the presence of valinomycin), in spite of the presence of a K^+ diffusion potential.

Additional information on possible mechanisms of action of fatty acids on glycine transport could be obtained from the efflux experiment presented in Table 5. Dilution of vesicles actively preloaded with ^{14}C glycine resulted in a spontaneous efflux that led to release, after 10 min, of up to 50 % of the accumulated amino acid (Table 5). The addition of arachidonic acid to the diluent caused a considerably greater loss of the accumulated glycine.

Fig. 3. Effect of arachidonic acid on $^{22}\text{Na}^+$ influx into plasma membrane vesicles

Plasma membrane vesicles from C6 glioblastoma cells were incubated for 15 min at 25 °C in the presence (○) or the absence (●) of arachidonic acid (0.25 nmol/ μg of protein). Transport of $^{22}\text{Na}^+$ was measured in a medium containing 0.4 μCi of $^{22}\text{Na}^+$ under the conditions described in the Experimental Section. Values represent means $\pm$ S.E.M. of three independent experiments.

Table 4. Effect of arachidonic acid on the electrochemical component of glycine uptake

Plasma membrane vesicles from C6 glioma cells preloaded with KCl medium were preincubated for 15 min at 25 °C in the absence or presence of 0.25 nmol of arachidonic acid/ μg of protein. Valinomycin was added 1 min before the transport assay started. The glycine transport assay was carried out for 15 min in the presence of 10 μM - ^{14}C glycine and an artificial Na^+ gradient (out > in). Uptake of ^{3}H tetraphenylphosphonium bromide (TPP) was carried out for 4 min in the presence of 0.2 μM - ^{3}H TPP. Values are the means $\pm$ S.E.M. of three replicate determinations. Values in parentheses represent % of control values in the absence of fatty acid. Values were compared with control values by using Student's *t* test: * *P* < 0.05; ** *P* < 0.01.

Addition	High-affinity glycine uptake (pmol/15 min per mg of protein)	TPP uptake (pmol/4 min per mg of protein)
None	210 $\pm$ 8 (100)	1.65 $\pm$ 0.04
Valinomycin (5 μM)	252 $\pm$ 6* (120)	2.05 $\pm$ 0.05
Arachidonic acid (0.25 nmol/ μg of protein)	125 $\pm$ 5** (60)	1.50 $\pm$ 0.07
Arachidonic acid + valinomycin	143 $\pm$ 5** (68)	1.99 $\pm$ 0.06

As shown in Table 6, the release of endogenous fatty acids by incubation with various concentrations of phospholipase A_2 caused inhibition of glycine transport. The removal of fatty acids from phospholipase A_2 -treated membranes by BSA resulted in a partial restoration of glycine transport.

The Arrhenius plots for the system of glycine transport in the presence or absence of arachidonic acid are depicted in Fig. 4. A discontinuity in the Arrhenius plot was observed in plasma membrane vesicles from the controls, with a break-point temperature of 21.0 ± 1.1 °C. The average apparent activation energy for the glycine transport system in the controls was found to be 10.0 ± 0.1 kJ/mol (2.40 ± 0.03 kcal/mol) above and 22.1 ± 0.4 kJ/mol (5.29 ± 0.09 kcal/mol) below the transition temperature. Although there is some scatter in the experimental points, no apparent discontinuity in the Arrhenius plot is shown in the presence of arachidonic acid, with the apparent activation energy in this case being 14.2 ± 0.1 kJ/mol (3.40 ± 0.03 kcal/mol). In membrane-bound reactions, the transition temperature, shown as a 'break' in Arrhenius plots, represents a phase change (gel-liquid crystal) in the membrane fluidity which is dependent on the temperature.

Table 5. Effect of arachidonic acid on the efflux of glycine

Plasma membrane vesicles from C6 glioblastoma cells were preloaded with ^{14}C glycine as indicated in the Experimental section and were diluted in NaCl medium. Efflux was allowed for 10 min with the indicated additions. Values are the means $\pm$ S.E.M. of three replicate determinations. Values in parentheses represent % of glycine retained in the absence of arachidonic acid at zero time of efflux. Values were compared with control values by using Student's *t* test: ** *P* < 0.01; *** *P* < 0.001.

Conditions	Addition	^{14}C Glycine retained (pmol/mg of protein)
Influx	None	337 $\pm$ 11 (100)
Efflux	None	163 $\pm$ 7** (48)
Efflux	Arachidonic acid (0.25 nmol/ μg of protein.)	69 $\pm$ 5*** (20)

Table 6. Effect of phospholipase A₂ on the high-affinity uptake of glycine

Plasma membrane vesicles from C6 glioblastoma cells were preincubated for 30 min at 30 °C in the presence of the indicated additions. After washing by centrifugation, the transport assay was carried out for 15 min at 25 °C in the presence of 10 μ M-[¹⁴C]glycine and an artificial NaCl gradient (out > in). Values are the means $\pm$ S.E.M. of three replicate determinations. Values were compared with control values by using Student's *t* test: * *P* < 0.05; ** *P* < 0.001.

Addition	High-affinity glycine uptake (pmol/15 min per mg of protein)
None	228 $\pm$ 10 (100)
BSA (5 mg/ml)	237 $\pm$ 4 (104)
Phospholipase A ₂ (2 units/ml)	187 $\pm$ 6* (82)
Phospholipase A ₂ (2 units/ml) + BSA	217 $\pm$ 8 (95)
Phospholipase A ₂ (4 units/ml)	109 $\pm$ 6** (48)
Phospholipase A ₂ (4 units/ml) + BSA	182 $\pm$ 9* (80)

DISCUSSION

The results given in the present paper clearly show that the high-affinity transport system for glycine in C6 glioma cells is inhibited by arachidonic acid and other unsaturated non-esterified fatty acids both under equilibrium conditions and when considering initial rates of transport. This inhibition was selective in two respects. First, the transport of leucine (transported by the Na⁺-independent L system) (Shotwell *et al.*, 1983) was only slightly affected by unsaturated fatty acids. Secondly, there is a correlation between the structure of the fatty acid molecule and the extent of inhibition of Na⁺-dependent glycine transport. It is worth noting that saturated fatty acids had little or no effect, and that at least one double bond was required to exert substantial

inhibition. Our results also indicate that, whereas the position of the double bond is not important, its configuration has a significant effect on the degree of inhibition. Clearly, fatty acids with the *cis* configuration are far more inhibitory than those with the *trans* configuration. Moreover, in the case of arachidonic acid, it was more inhibitory than its corresponding alcohol or methyl ester, suggesting that any change at the polar end of the fatty acid molecule also plays a role in the inhibitory effect. Such specificity, along with the reversibility by BSA, a protein that binds fatty acids, indicates that the inhibitory effect is not associated with a general detergent action on the membrane. These characteristics are similar to those described for fatty acid inhibition of the Na⁺-dependent amino acid uptake in synaptosomes (Peterson & Raghupathy, 1985).

Although the mechanism by which non-esterified fatty acids inhibit Na⁺-dependent amino acid transport is not known, unsaturated fatty acids have been reported to alter several membrane-associated cellular processes, such as changes in membrane fluidity and lipid packing (Usher *et al.*, 1978; Klausner *et al.*, 1980); depolarization of the membrane by increasing the permeability to Ca²⁺ (Troeger *et al.*, 1984); uncoupling mitochondrial oxidative phosphorylation (Heaton & Nichols, 1976); or uncoupling transport systems from the Na⁺ gradient (Rhoads *et al.*, 1983a). In order to distinguish between some of the above-mentioned mechanisms, we have performed most of the experiments in plasma membrane vesicles from C6 glioblastoma cells. This preparation enabled us to control accurately the ionic composition inside and outside the vesicles and to eliminate possible metabolic interferences. These vesicles are able to maintain artificially established Na⁺ gradients (Zafra & Giménez, 1986b) which act as the sole source of energy to drive the uptake of glycine under the experimental conditions established here. Glycine transport in plasma membrane vesicles has several features that are similar to the uptake in intact cells (Zafra & Giménez, 1986b), i.e. Na⁺-, Cl⁻- and $\Delta\psi$ -dependence.

The fact that inhibition occurred under the defined energetic states employed for vesicle transport eliminates some of the possible sites of action of unsaturated fatty acids. First, a non-specific effect of arachidonic acid on the integrity of the ionic gradients in our experimental conditions is excluded on the basis of measurements of membrane permeability to ²²Na⁺ in the presence of arachidonic acid. Secondly, the $\Delta\psi$ component of the transport system seems not to be responsible for the inhibition effect, as demonstrated by the fact that inhibition by arachidonic acid cannot be reversed in the presence of valinomycin. Moreover, the inhibition observed under equilibrium conditions, in which the isotope exchange is independent of ionic gradients, supports an effect on the carrier protein.

Previous results from our laboratory have shown that carrier-mediated efflux of glycine occurs from vesicles preloaded with the labelled amino acid (Zafra & Giménez, 1988). The efflux process of glycine has the same intravesicular ion requirements as the vesicular uptake. Thus the carrier system catalyses glycine transport in either the inward or the outward direction, with vectorial translocation determined by the direction of the Na⁺ or Cl⁻ gradient. The stimulation of the efflux of glycine by arachidonic acid, in spite of the presence of a Na⁺ gradient directed inward, suggests an uncoupling effect of fatty acids which converts the glycine/Na⁺ symport mechanism to a carrier-mediated diffusion system, in which glycine moves down its own chemical gradient out of the cell, as suggested by Rhoads *et al.* (1983b) in synaptosomes.

Although the origin of non-esterified fatty acids in brain is not clear, they could be produced by the action of endogenous phospholipases and/or acylglycerol lipases. Our results show that exogenously added phospholipase A₂ inhibits the uptake of

Fig. 4. Arrhenius plots of high-affinity glycine uptake in plasma membrane vesicles from glioblastoma cells

Uptake was measured in controls (■) and in the presence of arachidonic acid (0.30 nmol/ μ g of protein) (●). The vesicles were preincubated for 20 min in the presence of arachidonic acid. The transport assay was carried out for 1 min in the presence of 35 μ M-[¹⁴C]glycine. Each point is the mean $\pm$ S.E.M. of four experiments.

glycine in a BSA-reversible manner, suggesting that the release of fatty acids by this enzyme is responsible for the observed inhibition.

Abrupt changes in the slopes of Arrhenius plots at a particular temperature have been taken to represent a phase transition in the lipid environment of the membrane-bound enzymes and transport systems (Kunamoto *et al.*, 1971; Kimelberg & Papahadjopoulos, 1974; Nemat-Gorgani & Meisami, 1979; Brasitus & Schater, 1980; McElhaney, 1982; Stubes, 1983; Hanretta & Lombardini, 1987). In the present investigation, the thermal behaviour of the glycine uptake system in plasma membrane vesicles from glioblastoma cells changed dramatically in the presence of arachidonic acid, thus reflecting a perturbation of the membrane lipids in this situation, which could influence the lipid-protein interactions. Such interaction could also affect the conformation or aggregation state of the protein, and the resulting changes in protein structure with temperature might also determine changes in the Arrhenius plot, as has been emphasized by some authors (Thorneley *et al.*, 1975; Anzai *et al.*, 1978; Gómez-Fernández *et al.*, 1979).

All of these facts have led us to the conclusion that unsaturated fatty acids may act directly on the lipid domain surrounding the carrier in a way that affects its function. The differential effects of the non-esterified fatty acids presented in this paper are consistent with the results of Klausner *et al.* (1980), who demonstrated that non-esterified unsaturated fatty acids, especially arachidonic acid, readily intercalate into fluid domains of the membrane and produce significant changes in the packing of the lipid molecules.

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