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The effect of arachidonic acid and free fatty acids on vesicular uptake of glutamate and γ-aminobutyric acid

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Abstract

The manner in which arachidonic acid and other free fatty acids influence the vesicular uptake of glutamate and γ-aminobutyric acid (GABA) has been investigated. The *cis*-polyunsaturated fatty acid arachidonic acid (20:4), eicosapentanoic acid (20:5) and linolenic acid (18:3) at 150 nmol/mg protein (50 μM) inhibited the vesicular uptake of glutamate and GABA more than 70%. Reduced inhibition of vesicular uptake was seen with the *cis*-monounsaturated fatty acid oleic acid (18:1) and the *trans*-mono-unsaturated fatty acid elaidic acid (18:1). The saturated fatty acids stearic acid (16:0) and arachidic acid (20:0) had no significant effect on the uptake. The inhibition of vesicular uptake by arachidonic acid was prevented by the addition of fatty acid free bovine serum albumin. Arachidonic acid inhibited in a dose-dependent manner the generation of the transmembrane pH gradient of the synaptic vesicles. This inhibition was proportional to the inhibition of the vesicular uptake of glutamate and GABA. The saturated fatty acid arachidic acid showed no inhibition of ΔpH generation. Arachidonic acid at 200 nmol/mg of protein did not increase the uptake-independent leakage of glutamate and GABA from the vesicles, showing that the effect of arachidonic acid is not caused by an unspecific detergent effect. These results suggest that arachidonic acid and other polyunsaturated fatty acids are acting like proton-ionophores on the vesicular uptake of these neurotransmitters. This finding may have implications for the increased fatty acid concentration during pathological conditions like ischemia and in long term potentiation. © 1998 Elsevier Science B.V.

Keywords: Arachidonic acid; Fatty acid; Vesicular uptake; Glutamate; GABA (γ-amino-butyric acid)

1. Introduction

The concentrations of free fatty acids are relatively low under normal conditions in the mammalian brain. However, under various pathological conditions such as ischemia, hypoxia, hypoglycemia and seizures (Bazan, 1970, 1971; Agardh et al., 1980; Gardiner et al., 1981; Rehncrona et al., 1982; Yasuda et al., 1985) free fatty acids are known to accumulate. Under these adverse conditions large quantities of neurotransmitters are also being released (Benveniste et al., 1984). The two fatty acids released in the largest quantities are the polyunsaturated fatty acid, arachidonic acid and the saturated fatty acid, stearic acid. This accumulation of free fatty acids seems to result from an activation of phospholipases under the condition of tissue energy failure (Yasuda et al., 1985). The accumulation of free fatty acids could be harmful in several ways and can cause mitochondrial dysfunction (Wojtczak, 1976) and cellular edema (Chan and Fishman, 1978). Free fatty acids, particularly arachidonic acid, have also been shown to have a number of effects on neurotransmission in the central nervous system. Inhibition of transmitter uptake (amino acids, monoamines, choline (transmitter precursor)) into neuronal and glial cell preparations and synaptosomes by unsaturated fatty acids is well documented (Rhoads et al., 1982; Chan et al., 1983; Troeger et al., 1984; Yu et al., 1986, 1987; Boksa et al., 1988; Barbour et al., 1989; Zafra et al., 1990; Volterra et al., 1992a,b; Lundy and McBean, 1995; Trotti et al., 1995; Zerangue et al., 1995). Unsaturated fatty acids are also known to influence ion channels (Ordway et al., 1989, 1990) and the enzyme activities of Na^+/K^+-ATPase (Okun et al., 1992; Caspers et al., 1993) and protein kinase C (Hardy et al., 1994).

An increase of arachidonic acid is also observed during long term potentiation where it has been proposed to act as a retrograde messenger (Williams et al., 1989). Activation

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of postsynaptic glutamate receptors by glutamate or *N*-methyl-D-aspartate (NMDA) induce release of arachidonic acid from membrane phospholipids either directly, by activation of phospholipase A_2, or indirectly from degradation of diacylglycerol (Dumuis et al., 1988, 1990; Lazarewicz et al., 1988, 1992; Sanfeliu et al., 1990). In this context arachidonic acid has been shown to stimulate glutamate release from synaptosomes (Lynch and Voss, 1990; Herrero et al., 1992; Vázquez et al., 1994) probably by increasing the inositol phospholipid metabolism or activation of protein kinase C (Herrero et al., 1992; Hardy et al., 1994).

Glutamate and γ-aminobutyric acid (GABA) are among the most investigated neurotransmitters in the mammalian brain (Krnjevic, 1970; Fonnum, 1984, 1987) and the vesicular uptake of glutamate and GABA is well established (Disbrow et al., 1982; Naito and Ueda, 1985; Fykse and Fonnum, 1988; Maycox et al., 1988; Fykse et al., 1989; Kish et al., 1989). The vesicular uptake of these amino acid transmitters is driven by a transmembrane electrochemical proton gradient generated by a Mg^{2+}-ATPase. The uptake of glutamate is mainly driven by the membrane potential, whereas the uptake of GABA is driven by a combination of the membrane potential and the pH gradient (Shioi et al., 1989; Maycox et al., 1990; Shioi and Ueda, 1990; Tabb et al., 1992). Since free unsaturated fatty acids affect the uptake and release of neurotransmitters during several adverse conditions, we investigated their effect on vesicular uptake and storage of glutamate and GABA. The results show that arachidonic acid and other related long chained polyunsaturated fatty acids inhibit the vesicular uptake of both glutamate and GABA to the same extent. The inhibition is probably due to abolishing of the transmembrane vesicular electrochemical protongradient. The implications of these findings for the release of free fatty acids during neuropathological conditions such as ischemia and in long term potentiation are discussed.

2. Materials and methods

2.1. Preparation of synaptic vesicles

Synaptic vesicles were isolated as described by Fykse and Fonnum (1988). Shortly, a 10% (w/v) homogenate from brains of male Wistar rats (200–250 g) was made in 0.32 M sucrose, 10 mM Tris-maleate (pH 7.4) and 1.0 mM EGTA and centrifuged for 10 min at $800 \times g$ (3000 rpm; Sorvall SS-34 rotor). The pellet was removed and the supernatant centrifuged for 30 min at $20\,000 \times g$ (13 000 rpm; Sorvall SS-34 rotor) to give a crude synaptosomal fraction (P_2). The crude synaptosomal fraction was osmotically shocked by resuspension in 10 mM Tris-maleate (pH 7.4) and 0.1 mM EGTA and centrifuged for 30 min at $17\,000 \times g$ (12 000 rpm; Sorvall SS-34 rotor). The supernatant, containing vesicles, was subjected to 0.4 and 0.6 M sucrose density gradient centrifugation in a Contron TST 28.38 rotor at $65\,000 \times g$ (25 000 rpm) for 2 h. The vesicle fraction was isolated from the 0.4 M sucrose and stored in liquid nitrogen with no loss of activity.

2.2. Assay for vesicular uptake

Vesicular uptake of L-glutamate and GABA were determined as described by Fykse and Fonnum (1988). The incubation mixture contained, if not otherwise stated, 110 mM potassium tartrate, 10 mM Tris-maleate (pH 7.4) and 4 mM $MgCl_2$. Synaptic vesicles (0.07–0.1 mg of protein) were preincubated at 30°C for 15 min in absence or presence of the chosen fatty acid and the uptake was started by adding the substrate containing 1 mM L-[^{3}H]glutamate (1 μCi) or [^{3}H]GABA (1 μCi) and 2 mM ATP (disodium salt neutralized with Tris base). The mixture was incubated for 3 min and the reaction was stopped by adding 7.0 ml of ice-cold 0.15 M KCl, immediately followed by filtration through Millipore HAWP filters (diameters 25 mm and pore size 0.45 μm). The filters were washed two times with 0.15 M KCl before adding the filters to 10 ml Filter Count scintillation fluid (Packard). Filters were counted for retained radioactivity in a Packard Tri-Carb 2200 liquid scintillation spectrophotometer. All values were corrected for non-specific binding (blanks). Blanks were treated in the same way, but incubated on 0°C. The blank values for the uptake of GABA and glutamate accounted for 15–20% and 5–10% of the total uptake, respectively. Assays were carried out in duplicate. Protein measurements were performed according to Lowry et al. (1951).

Test agents were dissolved in absolute ethanol (stock solution 10 mM). The final concentration of ethanol in the incubation mixture did not exceed 1%, and ethanol had no significant effect on the vesicular uptake.

2.3. Assay for measurement of ΔpH

The ΔpH was determined by measuring the fluorescence quenching of acridine orange (Hell et al., 1990) using a Perkin Elmer Luminescence Spectrometer (LS 50 B). Excitation and emission wavelengths were 492 and 527 nm, respectively. Experiments were performed at 30°C in a medium containing 10 mM Tris-maleate (pH 7.4), 140 mM KCl, 4 mM $MgSO_4$ and 2 μM acridine orange. The reaction was started by the addition of ATP at 2 mM final concentration. The solution was continuously mixed using a magnetic stirrer and measurements were performed for 300 s. Inhibitors were added from stock solutions 3 min before addition of ATP in a volume of 1% of the assay volume. The specific protonophore carbonylcyanide-*p*-(trifluoromethoxy)phenylhydrazone (FCCP) (10 μM) was added at the end of the experiment to dissipate the pH gradient.

2.4. Statistical analysis

The results are expressed as mean ± S.E.M. values of absolute uptake or as relative uptakes (percentage of controls) from at least three separate experiments. Statistical analysis of each set of data was by Student's *t*-test.

2.5. Materials

Fatty acids, ATP (disodium salt), GABA and L-glutamate (dipotassium salt) were purchased from Sigma Chemical (St Louis, MO). [2,3-^{3}H]GABA (25–40 Ci/mmol) and L-[2,3-^{3}H]glutamate (17–25 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Male Wistar rats (150–200 g) were obtained from Møllegaard, Denmark.

3. Results

3.1. Inhibition of glutamate and GABA uptake by fatty acids

Arachidonic acid inhibited the vesicular uptake of glutamate and GABA to the same extent in a dose-dependent manner (Fig. 1). At 150 nmol arachidonic acid/mg protein (50 μM) the uptake of glutamate and GABA was inhibited by 75% and 62%, respectively. There was no significant difference in inhibition of glutamate and GABA ($P > 0.1$).

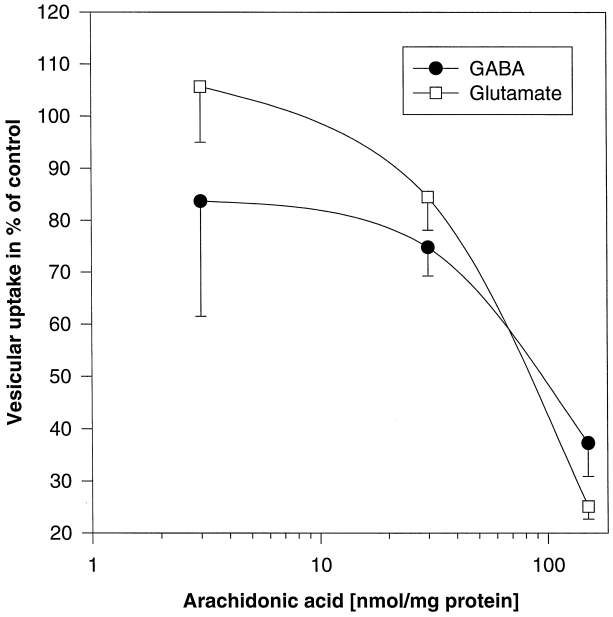

Fig. 1. Inhibition of vesicular [^{3}H]glutamate and [^{3}H]GABA uptake by arachidonic acid. Vesicles were preincubated for 15 min in the presence of different concentrations of arachidonic acid. The results are expressed in percentage of control. The rates of the uptake of glutamate and GABA in the absence of arachidonic acid were 1789 ± 182 ($n = 13$) and 509 ± 59 ($n = 5$) pmol/min/mg of protein, respectively. The results are the average ± S.E.M. of at least 3 separate experiments.

At 30 nmol arachidonic acid/mg protein only a slight inhibition (15–20%) was observed and in the presence of 3 nmol/mg protein, no inhibition observed. Decreasing the preincubation period in the presence of arachidonic acid from the standard 15 min to 4 min, did not significantly alter the results (results not shown). The effect of arachidonic acid on the uptake of glutamate was independent on the concentration of glutamate. Glutamate at 0.1 and 1 mM were compared and in both cases, 150 nmol arachidonic acid/mg protein caused the same level of inhibition (results not shown).

The effect of arachidonic acid on the uptake of glutamate is compared to the effect of other fatty acids and their derivatives (Fig. 2). The polyunsaturated fatty acids, eicosapentanoic acid (20:5) and linolenic acid (18:3), and the ethyl ester of arachidonic acid were all as effective as arachidonic acid in inhibiting the vesicular uptake of glutamate. The *trans*-unsaturated fatty acid, elaidic acid and the monounsaturated oleic acid, inhibited the uptake to a lesser extent, whereas the saturated stearic acid and arachidic acid gave no inhibition (Fig. 2).

3.2. Effect of bovine serum albumin on the inhibitory action of arachidonic acid

Higher concentrations of fatty acid free bovine serum albumin in the incubation mixture reduced the inhibitory effect of arachidonic acid on the vesicular uptake of glutamate (Fig. 3). When simultaneously adding 23 nmol/mg protein (0.03 mg) of bovine serum albumin and 150 nmol/mg protein arachidonic acid to the incubation mixture, the inhibition of vesicular uptake of glutamate decreased from 74% to 54%. The addition of 115 nmol/mg protein (0.15 mg) of bovine serum albumin to the medium fully prevented the inhibition of glutamate uptake by 150 nmol arachidonic acid/mg protein. Bovine serum albumin at 115 nmol/mg protein was able to prevent the inhibitory effect of arachidonic (200 nmol/mg protein) acid even if it was added 4 min after arachidonic acid (results not shown). When 115 nmol/mg protein of bovine serum albumin was preincubated with 300 nmol/mg of the saturated and noninhibitory fatty acid arachidic its ability to protect against inhibition by 150 nmol/mg arachidonic acid was significantly ($P < 0.05$) reduced (by approximately 30%) compared to control. Bovine serum albumin itself, at these concentrations, had no effect on the glutamate uptake.

3.3. Effects of arachidonic acid on efflux of glutamate and GABA from the vesicles

Synaptic vesicles were allowed to accumulate [^{3}H]glutamate and [^{3}H]GABA for 3 min in a standard uptake medium (see Section 2.2.) in the presence of ATP. The mixture was then diluted 20-fold by a ATP free and

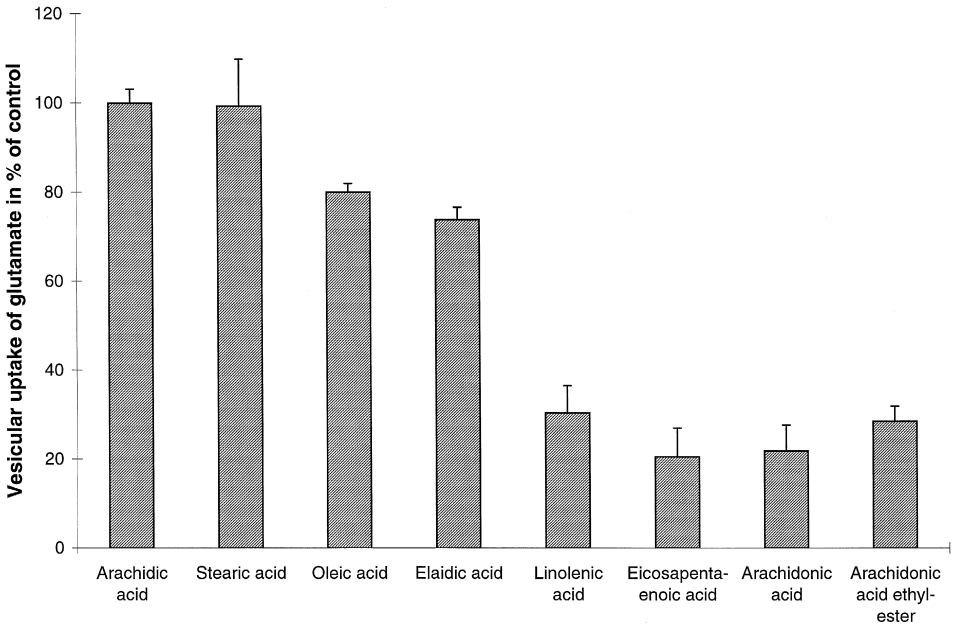

Fig. 2. Comparison of the effects of different fatty acids and arachidonate ethyl-ester on vesicular uptake of glutamate. Vesicles were preincubated for 15 min in the presence of 150 nmol fatty acids/mg of protein. The results are expressed as percentages of control (1999 ± 419 pmol/min/mg of protein ($n = 3$)) and are the mean ± S.E.M. of 3 separate experiments.

GABA or glutamate free medium, but in the presence or absence of arachidonic acid. The mixture was further incubated for 2 and 4 min before being terminated. Under these conditions uptake of glutamate or GABA does not occur. The results presented in Fig. 4, show, in agreement with Carlson and Ueda (1990) that at 30°C glutamate efflux occur at a significant rate after a 20-fold dilution of the standard uptake solution. The proportion of glutamate retained in presence of 200 nmol arachidonic acid/mg of

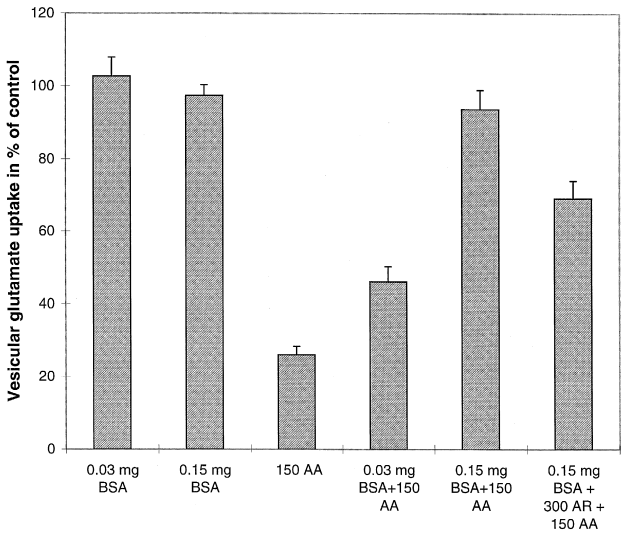

Fig. 3. The effect of fatty acid free bovine serum albumin on the reversibility of the arachidonic acid inhibition of vesicular [^{3}H]glutamate uptake. Vesicles (0.1 mg) were preincubated for 15 min in the absence (control) or presence of 0.03 mg bovine serum albumin (BSA), 0.15 mg bovine serum albumin, 150 nmol arachidonic acid (AA)/mg protein, 0.03 mg bovine serum albumin + 150 nmol arachidonic acid/mg protein, 0.15 mg bovine serum albumin + 150 nmol arachidonic acid/mg protein or 0.15 mg bovine serum albumin + 300 nmol arachidic acid (AR)/mg of protein + 150 nmol arachidonic acid/mg of protein before addition of 1 mM [^{3}H]glutamate and 2 mM ATP. The control value was 2504 ± 48 ($n = 5$) pmol/min/mg of protein. Each bar represents the mean ± S.E.M. of minimum 3 separate experiments.

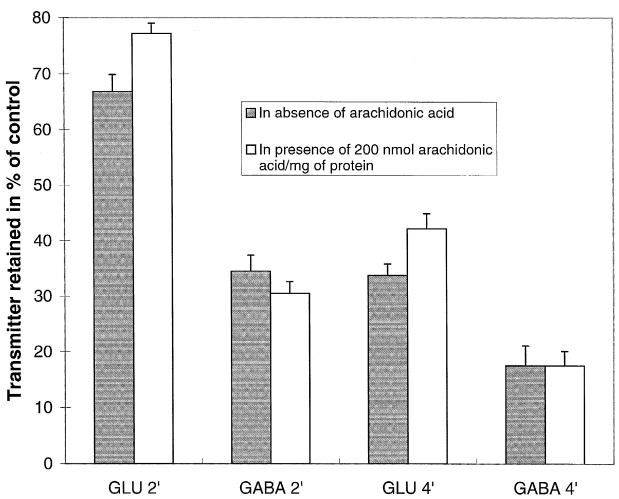

Fig. 4. Effect of arachidonic acid on uptake-independent glutamate and GABA efflux from [^{3}H]glutamate and [^{3}H]GABA preloaded vesicles (0.08 mg of proteins). Vesicles were allowed to accumulate [^{3}H]glutamate or [^{3}H]GABA for 3 min as before and then diluted 20-fold into a glutamate or GABA free medium. The amount of glutamate and GABA retained in % of control at 2 and 4 min and in absence or presence of 200 nmol arachidonic acid/mg of protein were measured. The control values for the uptake of glutamate and GABA were 3536 ± 386 ($n = 6$) and 957 ± 108 ($n = 6$) pmol/min/mg of protein, respectively. Bars represent mean of 3 separate experiments ± S.E.M.

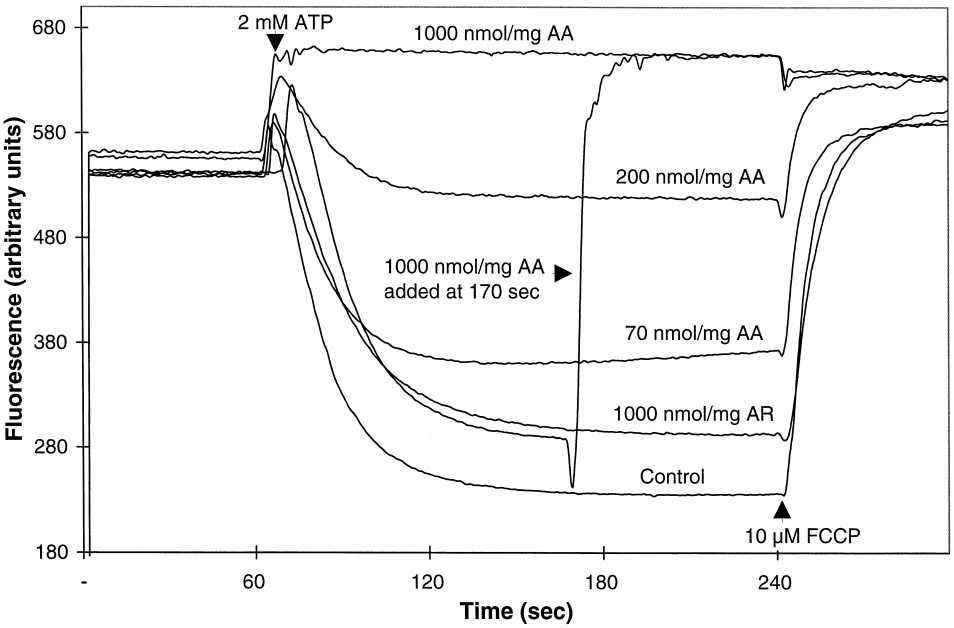

Fig. 5. The effect of arachidonic acid (AA) and arachidic acid (AR) on the ATP dependent generation of a transmembrane pH gradient in rat brain synaptic vesicles. Synaptic vesicles (0.08 mg of proteins) were preincubated in the presence of different concentrations of arachidonic acid and arachidic acid for 4 min at 30°C before the addition of 2 mM ATP. FCCP was added at the end as a control. The experiments were performed as described in Section 2.3.

protein was not significantly ($P > 0.05$) different from the proportion retained in the absence of arachidonic acid. Increasing the concentration of arachidonic acid to 1000 nmol/mg protein, increased the efflux of glutamate by 75%. The effect of arachidonic acid on the uptake-independent efflux of GABA was also studied (Fig. 4). GABA was lost after a 20-fold dilution of the incubation mixture at a much higher rate than glutamate (Fig. 4). As in the case of glutamate efflux, the GABA efflux was inhibited at 0°C (results not shown) and arachidonic acid at 200 nmol/mg protein did not significantly increase the efflux rate of GABA from synaptic vesicles ($P > 0.05$).

3.4. Effects of arachidonic acid on vesicular ΔpH

The effect of arachidonic acid on the ATP-dependent acidification of synaptic vesicles was studied. Two different assay systems were used. In one case, the arachidonic acid was preincubated with the synaptic vesicles for 4 min before addition of ATP. In the other case, arachidonic acid was added 100 s after the addition of ATP (Fig. 5). The results showed that arachidonic acid rapidly inhibited the generation of a transmembrane pH gradient. In this case the experimental volume was larger, but the amount of protein was the same as in the other experiments. The effect was immediate and dose-dependent. Arachidonic acid at 70 nmol/mg protein inhibited the generation of a transmembrane pH gradient by approximately 40%. A concentration of 200 nmol arachidonic acid/mg protein had the same inhibitory effect on ΔpH generation as on vesicular uptake of glutamate and GABA. Arachidonic acid at 1000 nmol/mg protein completely inhibited the generation of the pH gradient. A corresponding high concentration (1000 nmol/mg protein) of the saturated fatty acid arachidic acid gave almost no inhibition of the vesicular pH gradient.

4. Discussion

Arachidonic acid and other polyunsaturated fatty acids strongly inhibited the vesicular uptake of glutamate and GABA. On the other hand, mono-unsaturated and saturated fatty acids had little or no effect on the vesicular uptake of these transmitters. We believe that the more potent effect of the unsaturated fatty acids compared to the saturated fatty acids could be related to the lower melting point and therefore greater fluidity of the former. It has been demonstrated by Klausner et al. (1980) that free unsaturated fatty acids, especially arachidonic acid, readily intercalate into the membrane and produce significant changes in the packing of lipid molecules. Esterification of the hydrophilic and carboxylic 'head' of arachidonic acid did not significantly change the inhibition. Thus the fatty acids probably inhibit the vesicular uptake through the lipid phase and not through the water phase. This is different from the effect on the Na^+-dependent plasma membrane uptake of glutamate described by Trotti et al. (1995). They used liposomes reconstituted with the glutamate transporter GLT-1 and concluded that arachidonic acid inhibited the glial glutamate transporter directly from the water phase and not via the phospholipid membrane. Likewise protein kinase C was not activated by the ethyl ester of arachidonic acid (Holian et al., 1989; Herrero et

al., 1992). Arachidonic acid turned out to have a similar effect on the vesicular uptake of GABA and glutamate which indicate that arachidonic acid does not act directly on the transporter molecules. Different concentrations of glutamate in the incubation mixture did not influence the inhibitory effect of arachidonic acid. Thus arachidonic acid is not a competitive inhibitor of the vesicular uptake of glutamate. It is well known that the driving force for the vesicular uptake of transmitters is a transmembrane electrochemical proton gradient generated by a Mg^{2+}-ATPase (Naito and Ueda, 1985; Fykse and Fonnum, 1988; Fykse et al., 1989; Kish et al., 1989). Therefore, the effect of arachidonic acid on the generation of the transmembrane proton gradient was investigated. The polyunsaturated fatty acid, arachidonic acid, rapidly abolished generation of the vesicular pH gradient, whereas the saturated arachidic acid had no such effect. This means that unsaturated fatty acids may act like protonophores and cause equilibration of protons across the vesicle membrane and thereby destroy the electrochemical potential. This is also consistent with the effect of arachidonic acid on cultured hippocampal neurons (Wang et al., 1995). In that system arachidonic acid produce pH gradient changes similar to those elicited by the protonophore FCCP. Unsaturated fatty acids have earlier been shown to inhibit generation of a transmembrane proton gradient in chromaffine granule ghosts (Husebye and Flatmark, 1984) and in mitochondria (Heaton and Nicholls, 1976; Takeuchi et al., 1991). These properties may explain why arachidonic acid had a similar effect on the uptake of glutamate and GABA. The protonophores FCCP and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) inhibit vesicular uptake of glutamate and GABA to the same extent (Cidon and Shira, 1989; Fykse et al., 1989; Shioi and Ueda, 1990). However, if arachidonic acid caused a H^+–K^+ or H^+–Na^+ exchange like nigericin, the effect on the uptake of glutamate and GABA would have been different (Fykse and Fonnum, 1996). Carlson and Ueda (1990) have earlier reported that FCCP (10 μM) have a slight positive effect on the uptake-independent efflux of glutamate. We also could show an increased efflux of glutamate in the presence of a high concentration of arachidonic acid (1000 nmol/mg protein) but not at a lower concentration (200 nmol/mg protein). An unspecific detergent effect of the fatty acids causing large pores or burst of the vesicles is unlikely since neither glutamate nor GABA was lost from the vesicles when exposed to 200 nmol/mg protein of arachidonic acid. We also found that the inhibition of the vesicular uptake by arachidonic acid was reversed by the addition of 115 nmol/mg of fatty acid free bovine serum albumin which binds fatty acids with high affinity (Spector et al., 1969). Bovine serum albumin also prevents the effect of fatty acids on plasma membrane transport (Rhoads et al., 1982, 1983; Boksa et al., 1988; Lundy and McBean, 1995). Cerebral ischemia and other pathological adverse conditions are accompanied by a massive release of free fatty acids, specially arachidonic acid (Bazan, 1970; Rehncrona et al., 1982; Yasuda et al., 1985; Lazarewicz et al., 1992). Concentrations of free arachidonic acid in brain during cerebral ischemia have been reported to reach 65–500 nmol/g brain tissue (Rehncrona et al., 1982; Yasuda et al., 1985) and using a rough conversion factor of 1 ml volume/g tissue yields an arachidonic acid concentration of 65–500 μM. In our experiments 50 μM arachidonic acid (150 nmol/mg protein) is shown to inhibit the vesicular uptake of glutamate and GABA by 70–80%. The inhibitory concentrations in our system concur with the concentration range described in different systems (0.1–0.5 μmol/mg protein) (Rhoads et al., 1982; Troeger et al., 1984; Yu et al., 1986; Boksa et al., 1988; Zafra et al., 1990). The increased arachidonic acid level seen in pathological conditions may lead to an increased intracellular concentration both presynaptically and postsynaptically since such molecules are small, lipid soluble and can transverse membranes easily.

The inhibition of vesicular uptake of glutamate will ultimately lead to an increased level of presynaptic cytoplasmic glutamate. This increased level of cytoplasmic glutamate could be of importance under conditions of increased concentration of arachidonic acid such as ischemia or long term potentiation.

In conclusion, we have shown that arachidonic acid and other polyunsaturated acid inhibit the vesicular uptake of glutamate and GABA, probably by abolishing the pH gradient whereas unsaturated fatty acids had no such effect. The observed effect of arachidonic acid could be useful in understanding its role in long term potentiation and during adverse pathological conditions.

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