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Research report

Differential modulation of the glutamate transporters GLT1, GLAST and EAAC1 by docosahexaenoic acid

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Abstract

At present, the ability of polyunsaturated fatty acids (PUFAs) to regulate individual glutamate transporter subtypes is poorly understood and very little information exists on the mechanism(s) by which PUFAs achieve their effects on the transport process. Here we investigate the effect of *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA) on the activity of the mammalian glutamate transporter subtypes, GLT1, GLAST and EAAC1 individually expressed in human embryonic kidney (HEK) cells. Exposure of cells to 100 μM DHA increased the rate of D-[^{3}H]aspartate uptake by over 72% of control in HEK_{GLT1} cells, and by 45% of control in HEK_{EAAC1} cells. In contrast, exposure of HEK_{GLAST} cells to 200 μM DHA resulted in almost 40% inhibition of D-[^{3}H]aspartate transport. Removal of extracellular calcium increased the inhibitory potential of DHA in HEK_{GLAST} cells. In contrast, in the absence of extracellular calcium, the stimulatory effect of DHA on D-[^{3}H]aspartate uptake in HEK_{GLT1} and HEK_{EAAC1} cells was abolished, and significant inhibition of the transport process by DHA was observed. Inhibition of CaM kinase II or PKC had no effect on the ability of DHA to inhibit transport into HEK_{GLAST} cells but abolished the stimulatory effect of DHA on D-[^{3}H]aspartate transport into HEK_{GLT1} and HEK_{EAAC1} cells. Inhibition of PKA had no effect on the modulation of D-[^{3}H]aspartate transport by DHA in any of the cell lines.

We conclude that DHA differentially modulates the GLT1, GLAST and EAAC1 glutamate transporter subtypes via different mechanisms. In the case of GLT1 and EAAC1, DHA appears to stimulate D-[^{3}H]aspartate uptake via a mechanism requiring extracellular calcium and involving CaM kinase II and PKC, but not PKA. In contrast, the inhibitory effect of DHA on GLAST does not require extracellular calcium and does not involve CaM kinase II, PKC or PKA.

Theme: Neurotransmitters, modulators, transporters and receptors
Topic: Uptake and transporters

Keywords: Docosahexaenoic acid; Calcium; Glutamate; Transport; Kinase

1. Introduction

cis-4,7,10,13,16,19-docosahexaenoic acid (DHA) is the most highly unsaturated and one of the most abundant polyunsaturated fatty acids (PUFAs) in phospholipid fractions of mammalian brain [12]. It is a member of the n-3 series of PUFAs and is synthesized by phytoplankton, the plants at the base of the food chain for marine life. DHA is therefore abundant in fish, shellfish and marine mammals. Major interest into the role of DHA and other n-3 PUFAs in humans originally stemmed from the observation by Dyerberg and Bang [8] of lower incidences of cardiac arrhythmias in populations of Greenland Eskimos whose diets contain high levels of marine fat. This was shown to be a direct

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consequence of high levels of PUFAs such as DHA in the diet of these peoples [3,7]. It has subsequently been shown that PUFAs such as eicosapentaenoic acid (EPA) and DHA directly modulate various ion channels thus electrically stabilizing cardiac myocytes leading to lower incidences of cardiac arrhythmias [10]. Since these early observations, it has been shown that PUFAs can directly affect numerous cellular processes [19,31]. One such process is that of glutamate uptake in the brain. A family of high affinity sodium-dependent glutamate transporters (X_{AG}^-) are distributed throughout the brain and act to rapidly remove synaptically released glutamate (for review, see Danbolt, Ref. [6]). Failure to remove this neurotransmitter adequately can lead to damage and eventual death of neurons by a process termed excitotoxicity [18]. At least five subtypes of the mammalian glutamate transporter have been cloned so far [28,30]. These subtypes are GLT1, GLAST, EAAC1, EAAT4 and EAAT5. Reduced glutamate uptake has been observed in several neurodegenerative disorders such as Alzheimer's disease [17] and amyotrophic lateral sclerosis [26] thus indicating the importance of this reuptake process in normal brain function. Several reports have shown that fatty acids are capable of inhibiting high-affinity sodium-dependent glutamate uptake in various preparations [1,5,25,36]. The inhibition was due to the fatty acid itself, as inhibitors of fatty acid metabolism did not abolish the effect. Saturated fatty acids were ineffective and alteration of the fatty acid carboxyl group dramatically reduced the inhibitory potency of the fatty acid [25,35]. A potentiation of the inhibitory effect of highly unsaturated fatty acids such as arachidonic acid (ARA), EPA and DHA on synaptosomal glutamate uptake was observed when the fatty acid was pre-exposed to a purified synaptosomal preparation for 10 min prior to the initiation of the transport assay [2,13].

While the identification and cloning of the glutamate transporters has made it possible to study the function and regulation of individual transporter proteins, there have been very few reports investigating the effect of fatty acids on individual transporter subtypes. The first such study was by Zerangue et al. [38] who demonstrated a differential modulation of human glutamate transporter subtypes expressed in *Xenopus* oocytes and HEK cells by ARA. They showed that ARA activates the EAAT2 (GLT1) transporter subtype, but inhibits the EAAT1 (GLAST) subtype. The EAAT3 (EAAC1) transporter subtype was marginally activated by ARA [38].

We have recently shown that DHA is responsible for the greatest level of inhibition of synaptosomal D-[^{3}H]aspartate uptake compared to less highly unsaturated fatty acids such as ARA, EPA and linolenic acid [2]. The present study examines the effect of DHA on GLAST (EAAT1), GLT1 (EAAT2) and EAAC1 (EAAT3) individually expressed in HEK293 cells and shows that, like ARA, DHA can differentially modulate the activity of these glutamate transporter subtypes. The mechanism(s) underlying the effect of DHA is probed further using the calcium chelators BAPTA/AM and EGTA as well as selective inhibitors of CaM kinase II (KN-93), PKC (Bis-X) and PKA (H-89).

2. Materials and methods

2.1. cDNA cloning, plasmid construction and development of stable cell lines for GLAST, GLT1 and EAAC1

The coding sequences of the glutamate transporters GLAST, GLT1 and EAAC1 were isolated from complementary DNA (cDNA) derived from adult rat retina of both sexes by the polymerase chain reaction (PCR) as described previously [9,23,34]. The sense and antisense primer sequences were for GLAST: 5′-GAGAGCTAGCAAGTTCAAGACACTGAAGTGCA-3′ and 5′-GAGAGGGCCCGGGAGATGGACACATTGTAGA-3′, encoding for GLAST at positions 136-157 and 1886-1866 [33]; for GLT1: 5′-CACGCCATGGCATCAACCGAGGGTGCCAACAA-3′ and 5′-CCGCTCGAGAGAATTGGCTGAGAATCGGG-3′, encoding for GLT1 at position 93-121 and 1843-1824 [22]; for EAAC1: 5′-CCATCATGGGGAAGCCCACG-3′ and 5′-AGTCCCAGGCATCTAAGGCC-3′, encoding for the EAAC1 nucleotide sequences at positions 142–161 and 1724–1743 [11]. The PCR amplification product of GLAST, GLT1 and EAAC1 were agarose gel-purified, ligated into pBluescript KS-plasmid vector (Stratagene, La Jolla, CA), and transformed in *E. coli XL1*-Blue (Stratagene). Inserts from positive recombinants were sequenced on both strands and subcloned into the modified expression vector pBK-CMV (Δ[1098-1300]) (Stratagene), creating the plasmids pCMV-GLAST, pCMV-GLT1 and pCMV-EAAC1 which were used for functional analysis in a mammalian expression system.

2.2. Stable cell transfection and functional characterization

At 50–80% confluency, human embryonic kidney cell (HEK293, ATCC No. CRL 1573) cultures were transfected either with pCMV-GLAST, pCMV-GLT1, pCMV-EAAC1 or with pBK-CMV (Δ[1098–1300]) (40 μg/5 · 10^6 cells) as a control using calcium phosphate-mediated transfection as described previously [9,24,34]. 24 h post-transfection, the culture medium was replaced with fresh Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 50 μg/ml penicillin, 50 U/ml streptomycin, and 2 mg/ml Geneticin (Invitrogen) (G418 medium). The G418 medium was replaced every second day. Cultures with high glutamate uptake and high glutamate transporter protein expression were plated at low density in 1 mg/ml G418 media for additional 7 days. G418-resistant cells were chosen for cloning by limiting dilution. Briefly, cells were diluted to 1 cell/well in 96-well tissue culture plates and grown to confluency in 0.5 mg/ml G418 medium. Each clone was screened for maximal glutamate uptake, and stability of glutamate transporter expression (20 sub-culti-

vations). Cell lines – termed HEK_{GLAST}, HEK_{GLT1} and HEK_{EAAC1} – are routinely screened by PCR, Western blot analysis and uptake assays for the expression of GLAST, GLT1 or EAAC1.

2.3. Cell culture

Cells were grown in minimal essential medium (MEM) supplemented with 10% foetal bovine serum, 0.5 mg/L geneticin and penicillin/streptomycin on 100 mm diameter cell culture Petri dishes at 37 °C in a humidified atmosphere of 5% CO_2, 95% air. The cells were cultured as a monolayer and routinely passaged twice weekly.

2.4. Measurement of the rate of D-[^{3}H]aspartate uptake into HEK cell lines

Measurements of the rate of D-[^{3}H]aspartate uptake into all HEK cells were performed in 12 well plates. When 80% confluent, the cells were seeded at a density of 0.25×10^6 cells into each well of the 12 well plate. When these cells had reached 80% confluency (typically 2–3 days), they were used in D-[^{3}H]aspartate uptake assays. The growth medium was removed by suction and the cells were incubated with 450 μl Krebs' bicarbonate medium (contents in mM: NaCl 109.6, KCl 4.72, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25, glucose 11 and $CaCl_2$ 2.5 pH 7.4, gassed in 95% O_2/5% CO_2) containing fatty acid, drug or vehicle DMSO. The concentration of DMSO used in pre-incubation assays did not exceed 0.6% (v/v), which was a level that did not affect the rate of D-[^{3}H]aspartate transport into any of the cell lines. Following pre-incubation for the required time, the transport assay was initiated by the addition of 50 μl D-[^{3}H]aspartate solution (final concentration 10 μM, specific activity 185 Bq $nmol^{-1}$). After an incubation period of 6 min, the medium was removed by suction and the cell layer was washed twice with 500 μl Krebs' bicarbonate medium. The cells were then solubilized overnight in 500 μl 0.5 M NaOH. An aliquot of 300 μl of the solubilized cells was added to 5 ml scintillation cocktail (Ecoscint A) in a scintillation vial and the level of radioactivity incorporated into the cells was then determined by liquid scintillation spectroscopy. The remaining 200 μl aliquot of solubilized cells was retained for protein determination by the method of Bradford [4]. The amount of non-specific D-[^{3}H]aspartate binding to cell membranes and endogenous D-[^{3}H]aspartate uptake was determined by comparison of the data from transfected cells with HEK293 cells. The sodium dependency of D-[^{3}H]aspartate transport was assessed by using sodium-free Krebs' medium (choline chloride substituted for NaCl and 8.1 mM Tris used in place of $NaHCO_3$ to bring the pH to 7.4). The rate of sodium-dependent transport was calculated by subtraction of the rate of sodium-independent transport from the rate of total transport in the presence of sodium. Krebs' medium containing 1 mM EGTA in place of 2.5 mM $CaCl_2$ was used in calcium-free experiments.

Preliminary experiments (results not shown) demonstrated that the transport of D-[^{3}H]aspartate into both HEK_{GLT1}, HEK_{GLAST} and HEK_{EAAC1} cells followed Michaelis–Menten kinetics and that the rate of D-[^{3}H]aspartate uptake was linear from 0 to 15 min for HEK_{GLT1} and HEK_{EAAC1} cells and 0 to 20 min for the HEK_{GLAST} cells. Non-linear regression analysis of the data from these experiments was used to determine the kinetic characteristics (i.e., maximum velocity, V_{max}, and Michaelis constant, K_m) of each cell line. V_{max} values of 10.8 ± 0.7, 8.6 ± 1.3 and 5.4 ± 0.4 nmol mg protein^{-1} min^{-1} and K_m values of 343 ± 43, 158 ± 55 and 96.3 ± 22.0 μM were determined for D-[^{3}H]aspartate uptake into HEK_{GLT1}, HEK_{GLAST} and HEK_{EAAC1} cells, respectively.

Pre-incubation of HEK_{GLT1}, HEK_{GLAST} and HEK_{EAAC1} cells in serum-free medium for 1 h before measurement of transport had no effect on the quantity of D-[^{3}H]aspartate taken up in either the presence or absence of DHA (results not shown).

2.5. Materials

Docosahexaenoic acid (*cis*-4,7,10,13,16,19-docosahexaenoic acid) was purchased from Sigma Chemical Co., Poole, Dorset, UK and shipped on dry ice. The fatty acid was dissolved in 3% (v/v) dimethyl sulfoxide (DMSO) under a nitrogen atmosphere and stored under liquid nitrogen until required. Solutions of DHA were used within 1 month from the time of their preparation. D-[^{3}H]aspartate (specific activity 370–925 GBq (10–25 Ci) $mmol^{-1}$) was obtained from NEN (UK) LTD. KN-93 was supplied by Calbiochem, CN Biosciences, Nottingham, UK. Tissue culture reagents and plastics were purchased from Gibco BRL Life Technologies, Paisley, UK and Sarstedt LTD, Ireland. Bis-X and H-89 were provided by Sigma. All other reagents and chemicals were purchased from Sigma or Riedel-de Haën, Germany.

2.6. Statistical analysis

All transport experiments were conducted under conditions of initial velocity. The results in each figure are expressed as the mean ± SEM of the number of observations indicated. Data were analyzed by Student's unpaired *t* test (*). Where more than two comparisons were being made the Newman–Keuls multiple comparison test (δ) was used. In each case, the difference between means was considered significant at *P* values of less than 0.05.

3. Results

3.1. DHA differentially modulates GLT1, GLAST and EAAC1

Pre-incubation of HEK_{GLT1} or HEK_{EAAC1} cells with 100 μM DHA showed a time-dependent increase in

D-[^{3}H]aspartate transport. The greatest stimulation in transport in HEK_{GLT1} cells was seen at 20 min pre-incubation, at which time the rate of D-[^{3}H]aspartate uptake was increased to 172.7 ± 13.0% of control (Fig. 1). In HEK_{EAAC1} cells, the greatest stimulation of D-[^{3}H]aspartate transport was seen at 40 min pre-incubation (145.1 ± 7.2% of control). In contrast, pre-incubation of HEK_{GLAST} cells with 200 μM DHA showed a time-dependent decrease in D-[^{3}H]aspartate transport. The most significant decrease was observed at 10 min pre-incubation, at which time the rate of D-[^{3}H]aspartate uptake was decreased to 62.6 ± 5.3% of control (Fig. 1). In all cases, the effect was transient. Stimulation of transport in HEK_{GLT1} cells only occurred when the cells were pre-incubated with DHA for 18–25 min prior to initiation of the transport assay. DHA had no effect when pre-incubated for 0–12 min, or for greater than 30 min (Fig. 1). Stimulation of transport in HEK_{EAAC1} cells was significant between 25 and 40 min. DHA had no effect when pre-incubated for 0–20 min and was also ineffective at 60 min. On the other hand, DHA only caused significant inhibition of D-[^{3}H]aspartate transport in HEK_{GLAST} cells when pre-incubated for 8–10 min. Neither co-incubation (0 min pre-incubation) nor pre-incubation with DHA for periods greater than 12 min had any significant effect on the rate of D-[^{3}H]aspartate uptake in these cells (Fig. 1).

Further experiments with HEK_{GLT1}, HEK_{GLAST} and HEK_{EAAC1} cells showed that, in the case of HEK_{GLT1} cells, the threshold for stimulation of D-[^{3}H]aspartate uptake by DHA at 20 min pre-incubation occurred at a concentration of 50 μM (130.0 ± 6.8% of control), and that there was a concentration dependent stimulation in D-[^{3}H]aspartate uptake by DHA. At 200 μM DHA, the rate of D-[^{3}H]aspartate transport reached a level of 182.0 ± 8.5% of control (Fig. 2A). In contrast, the threshold for inhibition

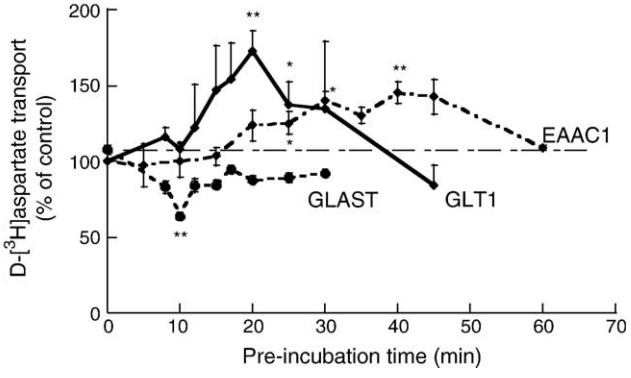


Fig. 1. Timecourse for the pre-incubation of HEK_{GLT1}, HEK_{GLAST} and HEK_{EAAC1} cells with DHA. The HEK_{GLT1} and HEK_{EAAC1} cells were pre-incubated with 100 μM DHA and the HEK_{GLAST} cells were pre-incubated with 200 μM DHA in Krebs' bicarbonate medium at 37 °C for 0–45 min (HEK_{GLT1}), 0–30 min (HEK_{GLAST}) or 0–60 min (HEK_{EAAC1}) prior to the initiation of the D-[^{3}H]aspartate uptake assay. The results are expressed as the % of control transport obtained in the absence of DHA and are the mean ± SEM of at least 4 individual determinations measured in triplicate. *P < 0.05, ***P < 0.001 compared to control with vehicle DMSO pre-incubated for the corresponding time.

of D-[^{3}H]aspartate transport by DHA in HEK_{GLAST} cells occurred at 100 μM (81.2 ± 2.2% of control), which was significantly lower than the level of inhibition observed with 200 μM DHA (Fig. 2B). In the case of HEK_{EAAC1} cells, a significant stimulation of D-[^{3}H]aspartate transport was observed with 10 μM DHA (177.0 ± 10.4% on control), which was not significantly different from the level of stimulation achieved with 100 μM DHA (166.0 ± 6.4% of control). Any further analysis of the concentration-dependency of the effect of DHA in any cell line was hindered by the limit of solubility of the fatty acid.

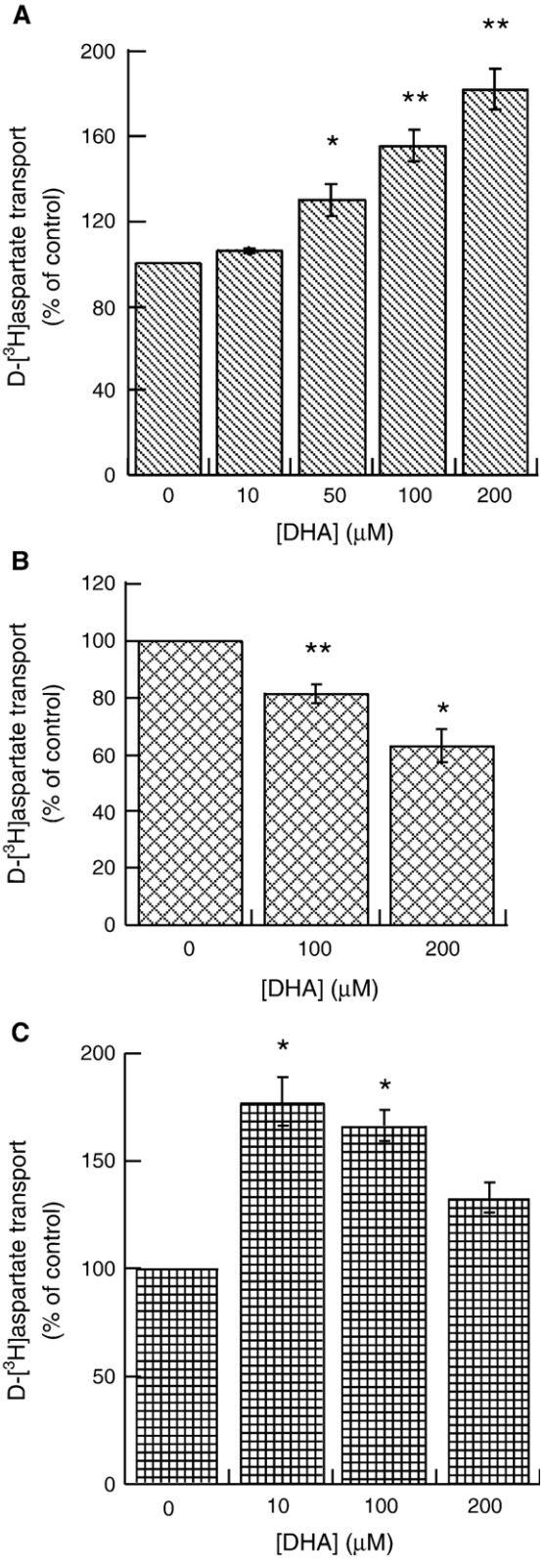
Kinetic analysis of D-[^{3}H]aspartate transport in HEK_{GLT1}, HEK_{GLAST} and HEK_{EAAC1} cells showed that, in each case, the maximum rate of transport (V_{max}) was affected by DHA. Pre-incubation of HEK_{GLT1} cells with 100 μM DHA for 20 min caused a significant increase in the V_{max} of D-[^{3}H]aspartate transport from 10.8 ± 0.7 nmol mg protein^{-1} min^{-1} in the absence of DHA to 21.0 ± 3.9 nmol mg protein^{-1} min^{-1} (P < 0.01) in its presence (Fig. 3A). Likewise, pre-incubation of HEK_{GLAST} cells with 200 μM DHA for 10 min decreased the V_{max} of D-[^{3}H]aspartate transport from 8.6 ± 1.3 nmol mg protein^{-1} min^{-1} in the absence of DHA to 5.9 ± 0.9 nmol mg protein^{-1} min^{-1} (P < 0.01) in its presence (Fig. 3B). Pre-incubation of HEK_{EAAC1} cells with 100 μM DHA for 40 min increased the V_{max} of D-[^{3}H]aspartate transport from 5.4 ± 0.4 nmol mg protein^{-1} min^{-1} in the absence of DHA to 7.0 ± 0.6 nmol mg protein^{-1} min^{-1} (P < 0.01) in its presence (Fig. 3C). There was no significant change in the K_m for D-[^{3}H]aspartate transport by DHA in any cell line.

Inclusion of the lipoxygenase inhibitor nordihydroguaiaretic acid (NDGA; 50 μM) or the cyclooxygenase inhibitor indomethacin (100 μM) had no effect on the action of DHA in HEK_{GLT1}, HEK_{GLAST} and HEK_{EAAC1} cells and methyl-DHA (100 μM) was inactive in all cell lines (results not shown).

3.2. Removal of extracellular calcium increases the inhibitory effect of DHA on GLAST and reverses the effect of DHA on GLT1 and EAAC1

The effect of extracellular calcium on the ability of DHA to modulate D-[^{3}H]aspartate transport in HEK_{GLT1}, HEK_{GLAST} and HEK_{EAAC1} was examined by pre-incubation of the cells in calcium-free Krebs' medium (Fig. 4). Removal of extracellular calcium had no effect on the control rate of transport in any cell line. The rate of D-[^{3}H]aspartate transport into HEK_{GLT1} cells pre-incubated in calcium-free medium in the presence of 100 μM DHA was reduced to 78.8 ± 1.3% of control (P < 0.005), while in the presence of 200 μM DHA, the level of transport was reduced further to 38.6 ± 2.8% (P < 0.001) (Fig. 4A). In the case of HEK_{GLAST}, a similar pattern was observed (Fig. 4B). In calcium-free medium pre-incubation of the cells with 100 μM DHA reduced D-[^{3}H]aspartate uptake to 53.6 ± 7.1% of control (P < 0.005) while pre-incubation with 200 μM DHA

decreased D-[^{3}H]aspartate uptake further to 27.9 ± 6.3% of control ($P < 0.005$). With HEK_{EAAC1} cells, pre-incubation of the cells with 100 μM DHA in calcium-free medium reduced D-[^{3}H]aspartate transport to 68.5 ± 4.6% of control ($P < 0.001$; Fig. 4C).



3.3. Reduction of the intracellular calcium concentration has no effect on the modulatory action of DHA in HEK_{GLT1}, HEK_{GLAST} or HEK_{EAAC1} cells

Experiments including the intracellular calcium chelator, BAPTA/AM, were performed to investigate the role of intracellular calcium in the modulatory activity of DHA on D-[^{3}H]aspartate uptake into HEK_{GLT1}, HEK_{GLAST} and HEK_{EAAC1} cells (Fig. 5). BAPTA/AM had no significant effect on the rate of D-[^{3}H]aspartate transport in HEK_{GLT1} cells either in the presence or absence of 100 μM DHA when pre-exposed to the cells for 20 min prior to the start of the transport assay (Fig. 5A). 100 μM DHA increased transport to 140.1 ± 1.5% ($P < 0.005$) and 155.3 ± 6.6% ($P < 0.0001$), respectively, in the presence and absence of 10 μM BAPTA/AM. A similar result was observed in HEK_{GLAST} cells (Fig. 5B). Pre-exposure of HEK_{GLAST} cells to BAPTA/AM for 10 min before the initiation of the transport assay had no effect on the level of inhibition mediated by 200 μM DHA. DHA on its own decreased D-[^{3}H]aspartate uptake to 57.3 ± 4.2% of control ($P < 0.05$), while the same concentration of fatty acid in the presence of 10 μM BAPTA reduced transport to 51 ± 1.2% ($P < 0.005$) of control. A similar result was also obtained with HEK_{EAAC1} cells (Fig. 5C): DHA on its own increased D-[^{3}H]aspartate transport to 152.0 ± 4.6% of control ($P < 0.05$), whereas the same concentration of DHA in the presence of 10 μM BAPTA/AM increased transport to 152.0 ± 9.1% of control ($P < 0.05$; Fig. 5C).

3.4. Inhibition of CaM kinase II or PKC has no effect on the inhibitory action of DHA in HEK_{GLAST} cells but abolishes the stimulatory effect of DHA in HEK_{GLT1} and HEK_{EAAC1} cells

The selective CaM kinase II inhibitor KN-93 was included in experiments to investigate whether this kinase is involved in the mechanism by which DHA alters the rate of D-[^{3}H]aspartate transport in HEK_{GLT1}, HEK_{GLAST} and/or HEK_{EAAC1} cells (Fig. 6). KN-93 on its own had no effect on the rate of D-[^{3}H]aspartate uptake in any cell line. In HEK_{GLT1} cells, the addition of KN-93 with 100 μM DHA to the incubation medium 20 min before the start of the transport assay abolished the increase in D-[^{3}H]aspartate transport that is caused by DHA alone (Fig. 6A). Under these conditions, the level of D-[^{3}H]aspartate uptake was 101.1 ± 3.3% of control compared to 149.8 ± 4.3% ($P < 0.05$) of control in the presence of 100 μM DHA. Inclusion

Fig. 2. Concentration dependence of the effect of DHA on D-[^{3}H]aspartate transport in HEK_{GLT1} (A), HEK_{GLAST} (B) and HEK_{EAAC1} (C) cells. Cells were incubated with DHA for 20 min (HEK_{GLT1}), 10 min (HEK_{GLAST}) or 40 min (HEK_{EAAC1}) prior to the initiation of the transport assay. The results are expressed as the % of control transport obtained in the absence of DHA and are the mean ± SEM of at least 4 individual determinations measured in triplicate. *$P < 0.05$, **$P < 0.01$ compared to control with vehicle DMSO pre-incubated for the corresponding time.

of KN-93 with 100 μM DHA in the incubation medium for 10 min before the start of the transport experiment had no effect on the level of DHA mediated inhibition of D-[^{3}H]aspartate uptake in HEK_{GLAST} cells (Fig. 6B). DHA on its own reduced the rate of transport to 69.1 ± 3.6% ($P < 0.005$) of control while in the presence of both DHA and KN-93 the level of D-[^{3}H]aspartate uptake was 61.6 ± 3.9% ($P < 0.001$) of control. Inclusion of KN-93 with 100 μM DHA in the incubation medium for 40 min before the start of the transport assay in HEK_{EAAC1} cells abolished the increase in transport observed in the presence of DHA alone (Fig. 6C). Under these conditions, the rate of D-[^{3}H]aspartate transport was 104.3 ± 11.8% of control compared to 153.0 ±0 4.5% of control ($P < 0.001$) in the presence of 100 μM DHA alone.

KN-93 had no effect on the timecourse for stimulation (HEK_{GLT1} and HEK_{EAAC1}) or inhibition (HEK_{GLAST}) of D-[^{3}H]aspartate uptake by DHA (results not shown). KN-93 was ineffective on its own at any of the timepoints investigated (0–30 min; results also not shown).

The selective PKC inhibitor, Bis-X (1 μM) had no effect on the rate of transport in each cell line in the absence of fatty acid (results not shown). In HEK_{GLT1} cells, the addition of Bis-X to the incubation medium with 100 μM DHA 20 min before the start of the transport assay abolished the increase in D-[^{3}H]aspartate transport observed in the presence of DHA alone (Fig. 6A). Under these conditions, the rate of transport was 110.3 ± 10.7% of control compared to 149.8 ± 4.3% of control ($P < 0.05$) in the presence of 100 μM DHA alone. In HEK_{GLAST} cells, Bis-X had no effect on the inhibition of transport observed with 200 μM DHA at 10 min pre-incubation (Fig. 6B). In HEK_{EAAC1} cells, the addition of Bis-X with 100 μM DHA to the incubation medium 40 min before the start of the transport assay abolished the increase in D-[^{3}H]aspartate transport that was observed in the presence of DHA alone (Fig. 6C). Under these conditions, the rate of D-[^{3}H]aspartate transport was 125.9 ± 11.6% of control, compared to 153.0 ± 4.5% of control ($P < 0.001$) in the presence of DHA alone. Bis-X had no effect on the rate of D-[^{3}H]aspartate transport in any of the cell lines in the absence of DHA (results not shown).

The PKA inhibitor, H-89 (1 μM) had no effect on the rate of D-[^{3}H]aspartate transport in any cell line in either the presence (Fig. 6) or the absence (results not shown) of DHA.

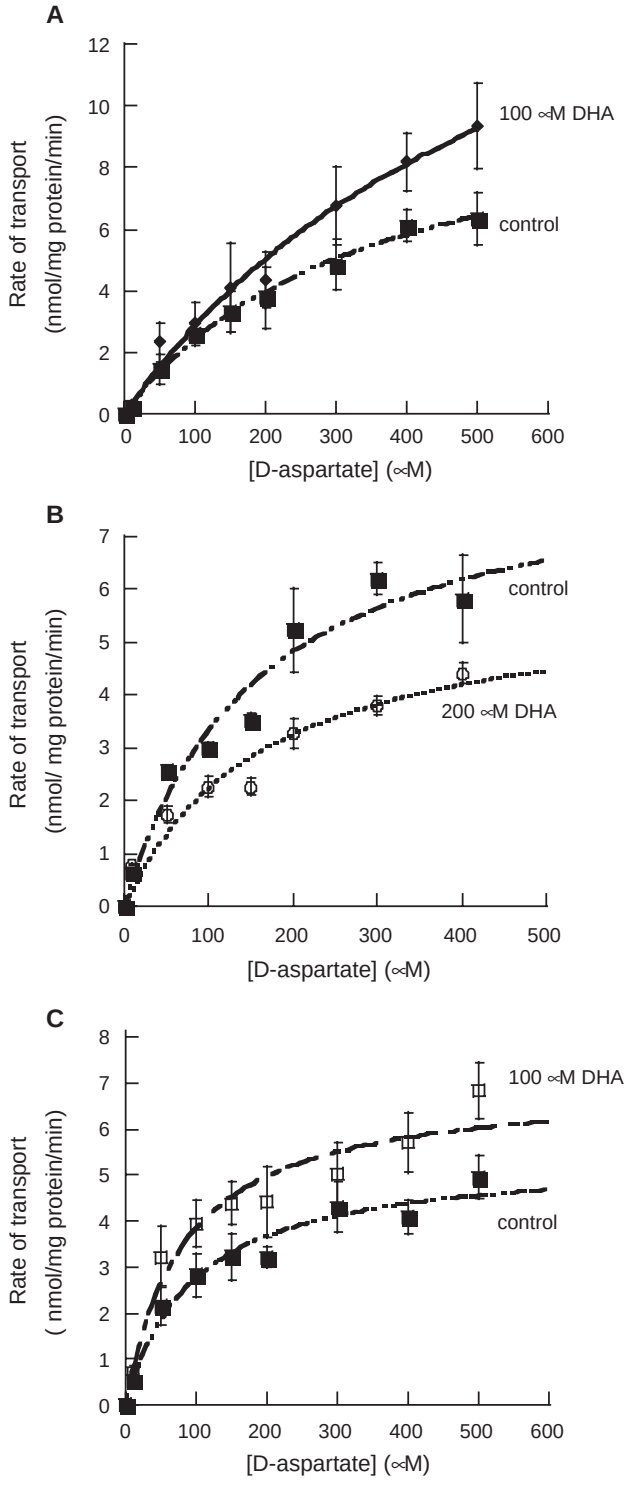


Fig. 3. Kinetic analysis of the effect of DHA on D-[^{3}H]aspartate transport in HEK_{GLT1} (A), HEK_{GLAST} (B) and HEK_{EAAC1} (C) cells. Cells were incubated in the presence of 100 μM DHA for 20 min (HEK_{GLT1}), 200 μM DHA for 10 min (HEK_{GLAST}) or 100 μM DHA for 40 min (HEK_{EAAC1}) prior to the initiation of the transport assay, which was performed over a concentration range of 1–500 μM D-[^{3}H]aspartate.

4. Discussion

It is clear from the results of experiments with HEK_{GLT1}, HEK_{GLAST} and HEK_{EAAC1} cells that DHA differentially affects these three transporter subtypes causing an increase of D-[^{3}H]aspartate uptake through GLT1 and EAAC1 while reducing the rate of uptake associated with GLAST. This differential effect is similar to that observed for ARA by Zerangue et al. [38], with the exception that, in their experiments, ARA only caused a minimal increase in D-[^{3}H]aspartate transport via EAAC1. The basis for this

difference may either be because, as we have previously shown, DHA is a more effective modulator of D-[^{3}H]aspartate transport than ARA [2], xor that the incubation period used in the experiments of Zerangue et al. [38] was not long enough for the fatty acid to achieve maximum stimulation of EAAC1-mediated transport. Nevertheless, it appears that DHA, like ARA, acts indirectly by a mechanism not involving alteration to the fluidity of the membrane. Inhibitors of lipoxygenase and cyclooxygenase enzymes have no affect on the ability of DHA to modulate D-[^{3}H]aspartate transport and methyl-DHA is inactive (data not shown). While it is obvious that DHA has opposing effects on GLAST, compared to GLT1 and EAAC1, it appears that the fatty acid acts transiently in all cases. It is also of interest that DHA must be pre-incubated with HEK_{GLT1} cells for 20 min, and with HEK_{EAAC1} for 40 min, in order for it to increase the activity of each transporter, whereas the inhibitory effect of the fatty acid on GLAST is greatest when it is pre-incubated with HEK_{GLAST} cells for just 10 min (Fig. 1). The precise reason for this difference is unknown, but it is possible that the upregulation of GLT1 and/or EAAC1 by DHA could involve increased expression of the transporter protein which would take longer than merely activation/inhibition of signaling molecules or interaction of the fatty acid with the transporter proteins, processes that may underlie DHA-mediated inhibition of GLAST.

It is worth noting that while 100 μM DHA was sufficient to almost abolish the uptake of D-[^{3}H]aspartate in synaptosomal experiments [2] the same concentration of DHA was much less effective in cell assays. It is also notable that the threshold concentration of DHA required to stimulate GLT1- or EAAC1-mediated transport of D-[^{3}H]aspartate (50 μM and 10 μM, respectively; Fig. 2) is less than the minimum concentration of DHA necessary to inhibit GLAST-mediated uptake. The reason for this discrepancy is not entirely clear, but it is known that cells grown in culture medium supplemented with foetal bovine serum are deficient in long chain PUFA [20]. It is therefore thought that a proportion of the exogenously added fatty acid may be sequestered for metabolic purposes. If this does occur, it would result in a lower concentration of fatty acid being available for modulation of the D-[^{3}H]aspartate uptake process. As a result of the diversion of added fatty acid to metabolic processes, the lower concentrations of fatty acid may become essentially inactive, which would explain why DHA is less potent in cell assays compared to the corresponding synaptosomal assays. In their study on the effect of ARA on individual glutamate transporter proteins, Zerangue et al. [38] have also used the higher fatty acid concentration of 100

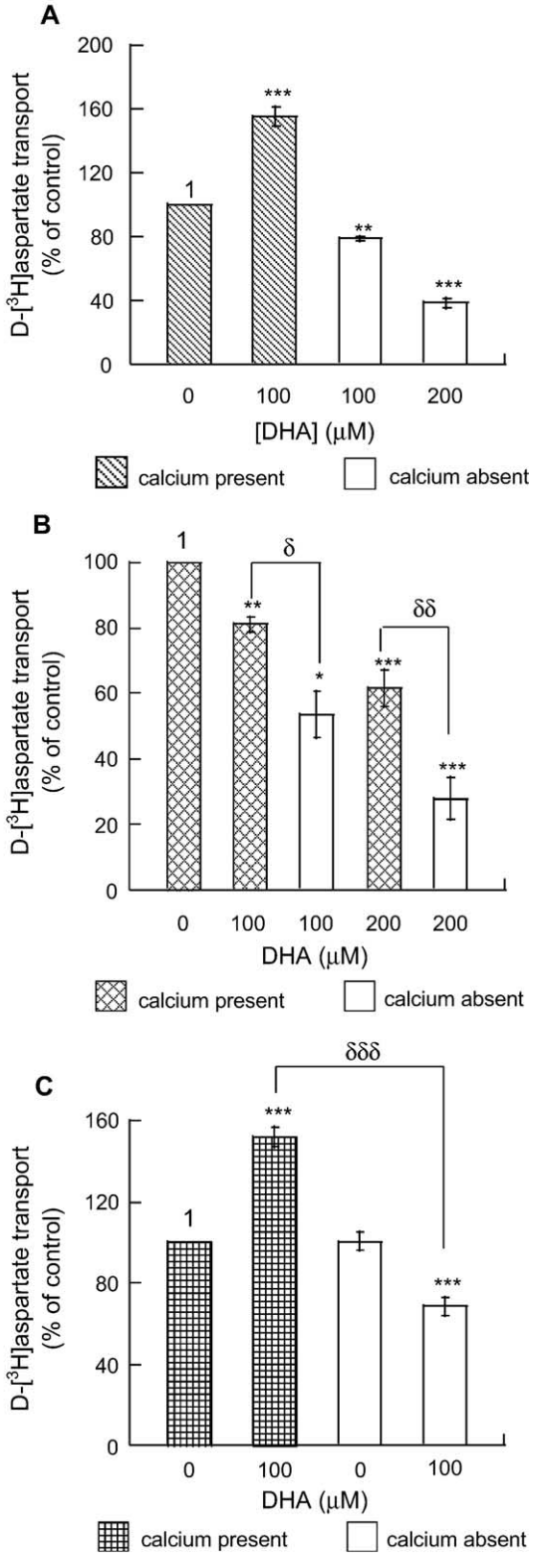
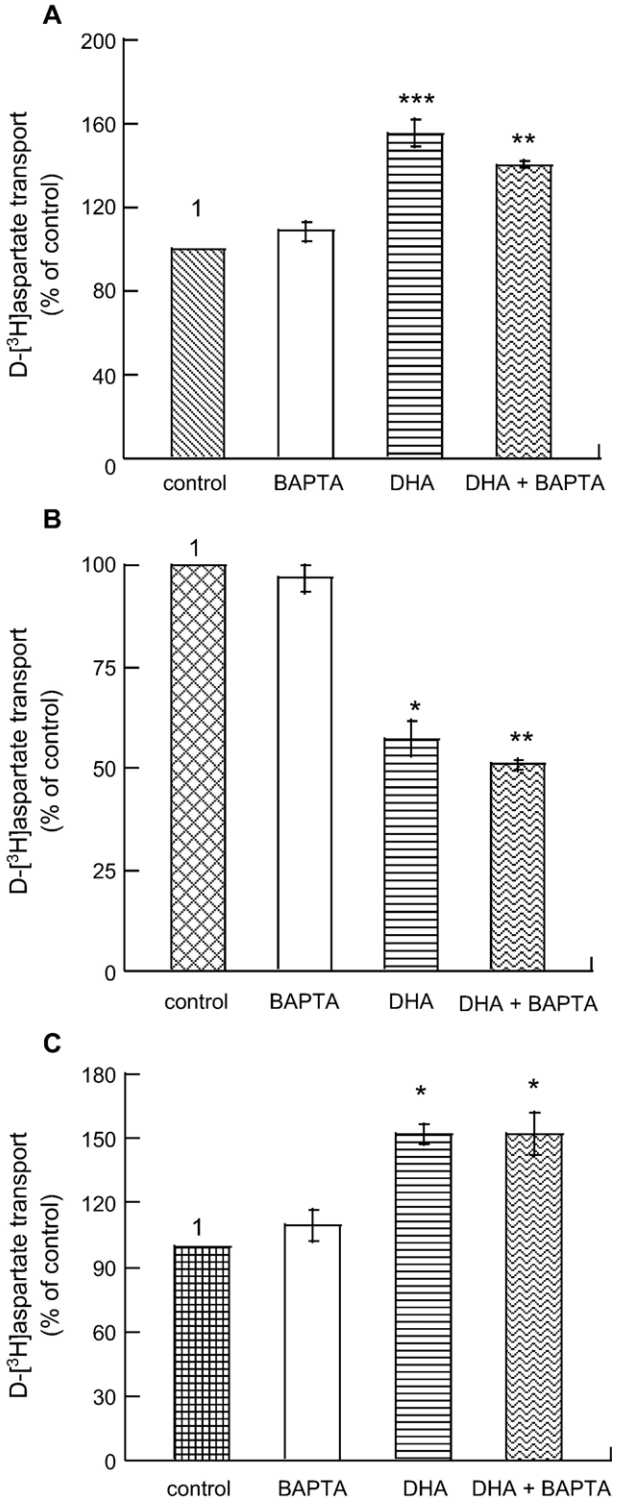


Fig. 4. Comparison of the effect of extracellular calcium on the ability of DHA to modulate the uptake of D-[^{3}H]aspartate into (A) HEK_{GLT1} (B) HEK_{GLAST} and (C) HEK_{EAAC1} cells. DHA at a concentration of 100 or 200 μM was pre-incubated with HEK_{GLT1}, HEK_{GLAST} or HEK_{EAAC1} cells in the presence (shaded bars) or absence (clear bars) of extracellular calcium in Krebs' medium at 37 °C for 10 min (HEK_{GLAST} cells), 20 min (HEK_{GLT1} cells) or 40 min (HEK_{EAAC1} cells) prior to the initiation of the uptake assay. The results are expressed as the % of control transport obtained in the absence of DHA (bar 1 in each plot) and are the mean ± SEM of at least 5 individual determinations measured in triplicate. *$P < 0.05$; **$P < 0.005$; ***$P < 0.0001$ compared to control with vehicle DMSO. *$P < 0.05$; **$P < 0.005$; ***$P < 0.001$ compared to corresponding pre-incubation in the presence of extracellular calcium.

μM. They have shown that 100 μM ARA increased the rate of L-[^{3}H]glutamate uptake into HEK cells expressing EAAT2 (the human homologue of GLT1) to 145.8% of control and reduced the activity of the EAAT1 (the human homologue of GLAST) transporter to 77.8% of control. In the results presented here, DHA caused similar effects for these two transporters.



Removal of calcium from the extracellular medium had no significant effect on the control rate of D-[^{3}H]aspartate transport in the absence of fatty acid in HEK_{GLT1}, HEK_{GLAST} or HEK_{EAAC1} cells (Fig. 4). Pre-incubation of HEK_{GLT1} or HEK_{EAAC1} with 100 μM DHA in calcium-free medium not only abolished the increase in the rate of D-[^{3}H]aspartate transport that was caused by this concentration of fatty acid in normal Krebs' medium but also resulted in a significant reduction in the rate of D-[^{3}H]aspartate accumulation. Increasing the concentration of DHA to 200 μM resulted in yet greater inhibition of D-[^{3}H]aspartate transport in calcium-free medium in HEK_{GLT1} cells. In the case of HEK_{GLAST} cells, the inhibitory potency of DHA was increased in calcium-free medium. From the results of these experiments, it appears that extracellular calcium is essential for the DHA-mediated increase in D-[^{3}H]aspartate transport observed in both HEK_{GLT1} and HEK_{EAAC1} cells. It also appears that the absence of extracellular calcium allows DHA to inhibit the uptake of D-[^{3}H]aspartate more strongly. This latter observation is similar to that observed in synaptosomes [2] and may be due to the absence of the chelation effect of calcium that is present in normal Krebs' medium.

The intracellular calcium chelator BAPTA/AM was also used in cell pre-incubation assays to determine what, if any, role intracellular calcium plays in DHA-mediated modulation of D-[^{3}H]aspartate uptake (Fig. 5). BAPTA/AM had no effect on the rate of transport in the absence of fatty acid in HEK_{GLT1}, HEK_{GLAST} or HEK_{EAAC1} cells and also had no effect on the action of DHA in all three cell types. These observations indicate that intracellular calcium is not required either for control transport or for DHA-mediated modulation of the transport process.

Inclusion of the selective CaM kinase II inhibitor KN-93, or the PKC inhibitor Bis-X, in pre-incubation assays had no effect on the control rate of D-[^{3}H]aspartate uptake in any cell line (results not shown). While KN-93 and Bis-X also had no significant effect on the inhibition of D-[^{3}H]aspartate transport in HEK_{GLAST} cells mediated by DHA, they both abolished the increase in D-[^{3}H]aspartate uptake observed when either HEK_{GLT1} or HEK_{EAAC1} cells were pre-incubated with DHA alone. This finding suggests that CaM kinase II and/or PKC have a functional role in the stimulatory effect of DHA on GLT1 and EAAC1. While DHA could also activate CaM kinase II or PKC in HEK_{GLAST} cells, this transporter subtype may not be affected

Fig. 5. The effect of the intracellular calcium chelator BAPTA/AM on D-[^{3}H]aspartate transport into (A) HEK_{GLT1} (B) HEK_{GLAST} or (C) HEK_{EAAC1} cells. The cells were pre-incubated with 10 μM BAPTA/AM alone or in combination with DHA in Krebs' bicarbonate medium at 37 °C for 10 (HEK_{GLAST}), 20 (HEK_{GLT1}) or 40 (HEK_{EAAC1}) min. In the case of HEK_{GLT1} and HEK_{EAAC1} cells, the concentration of DHA used was 100 μM, whereas 200 μM DHA was used in experiments with HEK_{GLAST} cells. The results are expressed as the % of control transport obtained in the absence of BAPTA/AM and DHA (bar 1 in each plot) and are the mean ± SEM of 5 individual determinations measured in triplicate. *$P < 0.05$; **$P < 0.005$; ***$P < 0.001$ compared to control with vehicle DMSO.

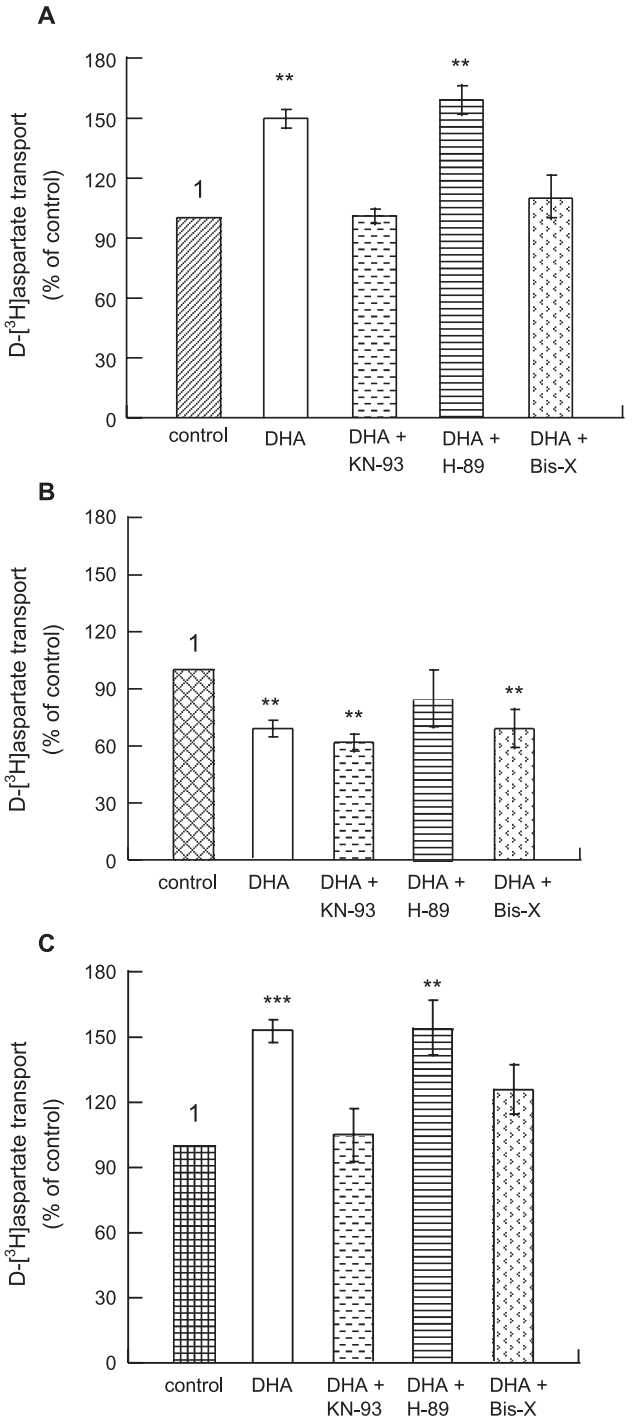


Fig. 6. The effect of selective kinase inhibition on D-[^{3}H]aspartate transport into (A) HEK_{GLT1}, (B) HEK_{GLAST} or (C) HEK_{EAAC1} cells. Each inhibitor was incubated for 20 (HEK_{GLT1}), 10 (HEK_{GLAST}) or 40 min (HEK_{EAAC1}) with 100 μM DHA (GLT1 and EAAC1) or 200 μM DHA (GLAST) prior to initiation of the transport assay. This inhibitor concentrations were 4 μM KN-93 (CamK II), 10 μM H-89 (PKA) or 1 μM Bis-X (PKC). The results are expressed as the % of control transport obtained in the absence of the both the inhibitor and DHA (bar 1) and are the mean ± SEM of 3 individual determinations measured in triplicate. $^{**}P < 0.005$; $^{***}P < 0.001$ compared to control with vehicle DMSO (KN-93 and Bis-X) or buffer (H-89).

by these kinases and so an inhibition of D-[^{3}H]aspartate transport is observed.

We suggest that DHA may exert opposing effects on both the GLT1 and EAAC1 transporter subtypes. The fatty acid may act to directly inhibit these transport proteins, while it may act indirectly via CaM kinase II and/or PKC to increase transporter activity. The observation by Trotti et al. [35], that ARA inhibits purified GLT1 reconstituted into artificial liposomes is a further indication that this transporter subtype can be inhibited by PUFA. The fact that this simple liposome system is devoid of intracellular signaling molecules and the inhibition is removed by treatment of the liposomes with BSA strongly suggests that the fatty acid inhibits this transporter subtype via a direct extracellular interaction either with the transporter protein itself or its immediate lipid environment. In the experiments presented here, the removal of extracellular calcium allows only the inhibitory effect of the fatty acid to occur while in the presence of extracellular calcium, DHA-mediated activation of CaM kinase II or PKC not only overcomes any DHA-mediated inhibition of the transport process but increases the rate of uptake beyond that observed in the absence of fatty acid. Perhaps, extracellular calcium is essential for the linkage of GLT1 to CaM kinase II or PKC, or it is required for DHA mediated activation of these kinases and so in the absence of the cation only DHA-mediated inhibition of the uptake process is observed. This would help explain not only how the positive effect of DHA on GLT1 and EAAC1 is lost in the absence of extracellular calcium, but also the inhibitory effect of the fatty acid that appears upon the removal of calcium from the extracellular medium. In the case of GLAST, the transporter protein may be affected much less, if at all, by either CaM kinase II or PKC, hence only the inhibitory effect of DHA is seen.

An enduring question that arises from this study is how the results reported here on the regulation of individual transporter subtypes by DHA relate to the net decrease in transport of D-[^{3}H]aspartate by DHA in synaptosomes [2]. While we do not have any information on the relative abundance of individual transporters in a synaptosomal preparation, it is obvious that the effect of DHA on HEK_{GLAST} cells is most similar to the effect of DHA observed in synaptosomes. This conclusion is based on the observation that DHA caused an inhibition of both synaptosomal and GLAST-mediated transport of D-[^{3}H]aspartate, and the inhibitory effect of DHA in both synaptosomes and HEK_{GLAST} cells was potentiated in the absence of extracellular calcium. However, since we believe that neither CaM KII nor PKC regulate the effect of DHA on GLAST, we suggest that the potentiation of inhibition of synaptosomal transport observed in the presence of KN-93 indicates that regulatory mechanisms associated with GLT1-mediated transport in synaptosomes favor net inhibition of uptake, whereas, in HEK cells, they favor a stimulation of transport. Further investigations into the regulation of D-[^{3}H]aspartate uptake by these transporters must be performed before any more comparisons can be

made with synaptosomal transport mechanisms. Calcium, both from intracellular stores [37] and the extracellular medium [14], is an important regulator of glutamate transport, and the association of this regulatory mechanism with that of DHA needs to be fully investigated.

Is becoming increasingly clear that modulation of glutamate transport by fatty acids is much more complex than had been previously anticipated. Simple mechanisms such as alterations to membrane fluidity cannot adequately explain the differential effects of fatty acids on different glutamate transporter subtypes. It is perhaps not surprising that such an important cellular process as glutamate transport should be subject to complex regulation by PUFAs. Modulation of glutamate transporters by DHA and other PUFAs is a potentially crucial mechanism for the regulation of neuronal signaling by this neurotransmitter. Activation of glutamate receptors has been shown to cause the release of free PUFA from both neurons [32] and glial cells. The fact that fatty acids can differentially modulate transporter subtypes could allow fine-tuning of neuronal activity. Indeed, it has been observed that ARA is a more potent inhibitor of glutamate uptake in the cerebellum and spinal cord than in the hippocampus [15]. The regional distribution of glutamate transporter subtypes that are differentially modulated by DHA may therefore lead to discrete differences in the pattern of neuronal activity throughout the brain. Depending on the transporter subtype, the released PUFA may act as a switch to limit the effect of glutamate by activating the transporter, or it may lead to an increase in synaptic efficacy by inhibiting the uptake of the neurotransmitter. Inhibition of uptake may also lead to the development of excitotoxicity. This excitotoxicity, which is caused by the persistent activation of glutamate receptors, is believed to be involved in a number of neurodegenerative disorders [16,21,27]. While upregulation of transport could limit the effect of glutamate by removing it from the vicinity of glutamate receptors, inhibition of the transport process could initiate or exacerbate an excitotoxic insult. The ability of PUFA to differentially modulate glutamate transporter subtypes, coupled with the fact that high levels of free PUFA have been observed in various disease states [29] suggests that PUFA-mediated modulation of the glutamate uptake process may be an important factor in the progression or limitation of damage caused by high concentrations of extracellular glutamate. Manipulation of the level of free PUFA in neurodegenerative disorders, or under conditions of ischemia or brain trauma may not only limit the neuronal destruction that is characteristic of these conditions, but may also be neuroprotective by increasing the uptake of extracellular glutamate.

An understanding of precisely how DHA and other PUFAs achieve their effects on glutamate transporters is therefore vital not only for our understanding of normal neurotransmission but may also lead to the development of therapies aimed at preventing or limiting neuronal damage in various neurodegenerative disorders.

Acknowledgments

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