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The effect of omega-3 fatty acids on Ca-ATPase in rat cerebral cortex

S. D. Kearns, M. Haag

Department of Physiology, University of Pretoria, Pretoria, South Africa

Summary Neuronal Ca-ATPase has the essential function of keeping intracellular Ca levels in the micromolar range. This is a prerequisite for normal neurotransmission. This study was designed to determine whether Ca-ATPase is a target for docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) action: results show that both these fatty acids are inhibitors of Ca-ATPase activity in synaptosomal membranes isolated from rat cerebral cortex ($-65 \pm 5\%$ at [DHA]=20 μg/ml, $-59 \pm 7\%$ at [EPA]=20 μg/ml). The inhibition caused by EPA, but not that of DHA, could be reversed completely by the addition of calphostin, a protein kinase C blocker. In contrast, DHA could stimulate Ca-ATPase activity ($+132 \pm 5\%$ at [DHA]=30 μg/ml) only in calmodulin-depleted membranes. In addition, Na,K-ATPase (which drives the Na–Ca exchanger) was inhibited by both DHA and EPA, both at 30 μg/ml ($-15 \pm 0.7\%$ and $-42 \pm 1\%$, respectively). These results suggest a mechanism that explains the dampening effect of omega-3 fatty acids on neuronal activity. © 2002 Elsevier Science Ltd. All rights reserved.

INTRODUCTION

Reports on the use of omega-3 fatty acids in the treatment of psychiatric disorders such as bipolar disorder[1] and schizophrenia[2] are increasing. Neuronal Ca-ATPase plays a vital role in the prevention of Ca toxicity[3,4] in nerve cells by extrusion of excess intracellular calcium, thus maintaining intracellular calcium homeostasis. Modulation of this enzyme could contribute to the possible mechanisms by which omega-3 fatty acids exert their beneficial effects. A membrane Na–Ca exchanger also contributes to Ca extrusion from the neuron,[3] the activity of this protein is driven by membrane Na,K-ATPase. Omega-3 fatty acids have been shown to have modulating effects on Ca-ATPase in a number of tissue cells, including colon,[5] cardiac myocytes,[6] T cells[7] and also in cortical microsomes.[8] Na,K-ATPase is also a well-known target for these fatty acids.[9,10] The lack of current data on the relationship between omega-3 fatty acids and ATPases influencing calcium transport in the neuron has prompted the following investigation.

One possible omega-3 fatty acid action mechanism is a direct interaction with Ca-ATPase: polyunsaturated fatty acids have been shown to mimic the direct activating effects of calmodulin on the enzyme.[11] It has also been suggested that omega-3 fatty acids induce mood-stabilising effects via the inhibition of neuronal protein kinase C,[12] and since the C terminal of the plasma membrane Ca-ATPase enzyme contains a regulatory site for protein kinase C,[13] this could be a possible mechanism by which modulating changes are produced.

The following study investigates the influence of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) on Ca-ATPase and Na,K-ATPase activity in synaptosomal membranes isolated from rat cerebral cortex. Possible mechanisms by which these fatty acids exert their effects on neuronal Ca homeostasis are addressed. This may contribute to current research in omega-3 fatty acid supplementation and psychiatric disorders.

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Correspondence to: Prof. Marianne Haag, Department of Physiology, University of Pretoria, P.O. Box 2034, Pretoria 0001, South Africa.
Tel.: +27-12-420-2537; Fax: +27-12-420-4483;
E-mail: mhaag@medic.up.ac.za

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MATERIALS AND METHODS

Reagents

DHA, EPA, calphostin (CP), genistein (G), ouabain, ethyleneglycol-bis-(β-aminoethyl ether) *N,N′*-tetraacetic acid (EGTA), Tris-ATP and dithiothreitol (DTT) were purchased from Sigma Chemicals. All other chemicals were obtained either from Sigma or Saarchem, Johannesburg.

Preparation of synaptosomal membranes from rat cerebral cortex

The preparation of cerebral cortex membranes was based on methods by Jones and Matus[14] and Petrali and Sulakhe[15] as described by Haag et al.[16] Briefly, male Sprague–Dawley rats, weighing between 200 and 250 g, were decapitated, slivers of cerebral cortex removed and homogenised. The homogenate was centrifuged at 1000*g* for 10 min at 4°C. The pellet was discarded and the supernatant centrifuged at 10 000*g* for 20 min at 4°C. This crude mitochondrial pellet was suspended in a hypotonic medium for 30 min for lysis to occur. The sample was then sonicated, and 59% sucrose-buffer solution was added to give a final sucrose concentration of 41.7%. A sedimentation-flotation technique was utilised to fractionate this preparation on a discontinuous sucrose gradient containing 10, 32 and 41.7% sucrose. After centrifugation at 60 000*g* for 110 min at 4°C, a white band of purified synaptosomes at the interface between the 41.7 and 32% sucrose solutions was removed. The membranes were pelleted by centrifugation at 40 000*g* for 30 min. The pellet was resuspended in 10.7% sucrose–10 mM imidazole–0.2 mM DTT (ISD) buffer solution. Aliquots were stored at −70°C until use in the enzyme assay.

Calmodulin-depleted membranes were obtained by suspending the final membrane pellet (see above) in 1 ml 50 mM imidazole–5 mM EGTA and incubating at 37°C for 20 min. The membranes were pelleted by centrifugation at 40 000*g* for 30 min at 4°C, washed, resuspended in ISD buffer, and stored at −70°C.

The influence of DHA and EPA on ATPase activities[17]

DHA and EPA were dissolved in ethanol and then diluted 1:400 with ISD buffer to give a final concentration of 250 μg fatty acid/ml. This solution was sonicated twice with a Vibra Cell sonicating apparatus (Sonics & Materials Inc., Danbury, CT, USA) for 10 s at 4°C before adding to the assay. Synaptosomes from rat cerebral cortex were pre-incubated with the sonicated fatty acid and all other reactants except ATP for 5 min at 37°C. Tris-ATP was added to start the 10 min enzyme reaction. The final concentrations in the Ca-ATPase reaction mixture were: 1 mM EGTA, 0.991 mM $CaCl_2$ (a buffer system giving rise to 0.1 μM free Ca^{2+}),[18] 52 mM imidazole–HCl, 3 mM $MgCl_2$, 64 mM sucrose, 0.04 mM DTT, 1 mM ouabain, 10–30 μg/ml fatty acid, 0.05% ethanol, and synaptosomes at a protein concentration of 130–200 μg/ml. The total incubation volume was 250 μl. Controls contained no $CaCl_2$ and Na,K-ATPase is inhibited by ouabain in this assay. The Na,K-ATPase assay was conducted in the same way except that test samples contained 100 mM NaCl, 20 mM KCl, no ouabain and no $CaCl_2$. Na,K-ATPase controls were the same as for Ca-ATPase. The reaction in both ATPase assays was stopped with 400 μl of 8.3% cold trichloroacetic acid (TCA). Zero blank samples were obtained by adding TCA before adding ATP.

Quantification of inorganic phosphate liberated by ATPase was done as follows: Samples were incubated at 45°C for 20 min with 1.5 ml ascorbic acid/molybdate to form a blue phosphomolybdate complex.[19] The samples were then centrifuged at 800*g* for 10 min at 4°C. Absorbance was measured at 820 nm. Enzyme activity was expressed as nmol Pi liberated/mg protein/min. Enzyme activities of controls (which represent Mg^{2+} and also basal, unspecific ATPase activities) were subtracted from those of the test samples.

The influence of calphostin and genistein on fatty acid action

CP (a protein kinase C blocker) and G (a tyrosine kinase blocker) were dissolved in DMSO. They were included in the pre-incubation step described above. The final concentrations of CP, G and DMSO in the enzyme assay were 0.5 μM, 50 μM and 0.05%, respectively.

RESULTS

Both inhibitory and excitatory effects of polyunsaturated fatty acids on membrane ATPases are known, a variety of action mechanisms have been suggested.[4–11] In the present study, DHA caused significant depression in synaptic membrane Ca-ATPase activity in a dose-dependent manner (range 10–30 μg/ml) as shown in Figure 1. The fatty acid concentrations used in this study[20,21] are within the normal range, but it is difficult to determine how much fatty acid is actually presented to the neuronal membrane.

Protein phosphorylation is an important mechanism by which Ca-ATPase activity is regulated,[12] and polyunsaturated fatty acids are known to modulate the activity of several protein kinases.[22,23] Neither CP (a protein kinase C inhibitor) nor G (a tyrosine kinase inhibitor) could reverse inhibition by DHA, thus ruling out activation of protein kinase C and tyrosine kinase as DHA second messenger pathways (Fig. 2), and suggesting alternative

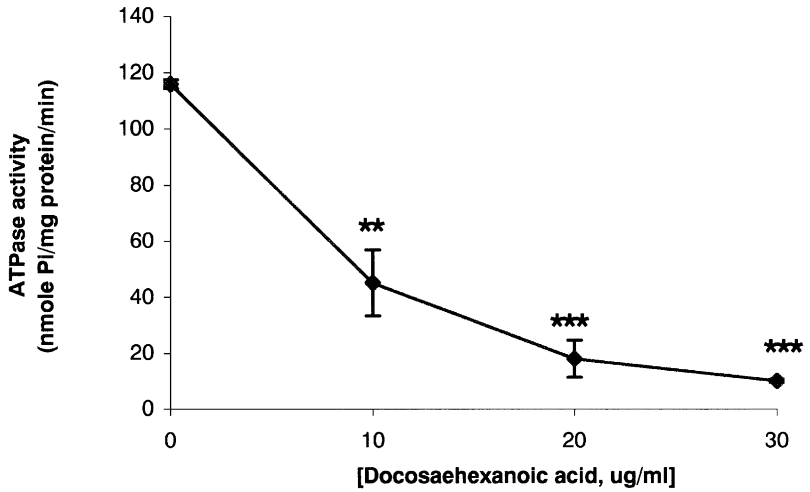

Fig. 1 Dose–response curve of the effect of DHA on Ca-ATPase in synaptic membranes (SMs) from rat cerebral cortex. After a 5-min pre-incubation period of SM with DHA, ATP was added to start a 10 min enzyme reaction. Final concentrations of DHA and SM protein in the reaction mixture were 10–30 μg/ml and 200 μg/ml, respectively. The mean ± SEM was calculated from a minimum of three independent observations. Comparing each individual DHA-treated value with the control value, *P* values were calculated according to Student's *t*-test (**$P < 0.01$, ***$P < 0.005$).

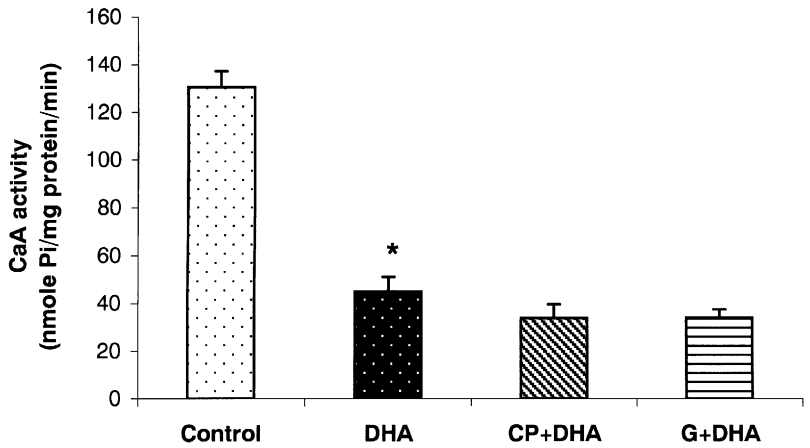

Fig. 2 The influence of the protein kinase C blocker CP and the tyrosine kinase blocker G on DHA inhibition of SM Ca-ATPase activity. After a 5-min pre-incubation of SM with DHA and CP or G, ATP was added to start the 10 min enzyme reaction. Final concentrations of DHA, CP, G and synaptic membrane protein in the reaction mixture were 20 μg/ml, 0.5 μM, 50 μM and 150 μg/ml, respectively. The mean ± SEM was calculated from a minimum of three independent observations. The data were analysed by one-way ANOVA and *P* calculated by Tukey's test. The activity of DHA-treated samples differed significantly from untreated controls (*$P < 0.05$). Neither CP nor G could reverse inhibition by DHA significantly.

means by which DHA carries out its depressive effects. When synaptic membranes were depleted of calmodulin, a significant stimulatory effect of Ca-ATPase by DHA (30 μg/ml) could be observed. Since CP could not reverse this effect, the DHA-induced activation of Ca-ATPase did not take place via phosphorylation by protein kinase C (Fig. 3).

EPA (20 μg/ml) also produced significant inhibitory effects in Ca^{2+}-ATPase activity (Fig. 4). In contrast to DHA, CP could reverse this inhibition, indicating a second messenger pathway via protein kinase C for EPA.

The Na–Ca exchanger in the neuronal membrane is driven by Na,K-ATPase: DHA and EPA (both at a concentration of 30 μg/ml) also produced significant depressive effects on this enzyme, as shown in Figure 5.

DISCUSSION

Several authors have reported on the action of poly-unsaturated fatty acids on Ca-ATPase: EPA feeding activated Ca-ATPase in brain microsomes that had previously been suppressed by cholesterol supplementation,[8]

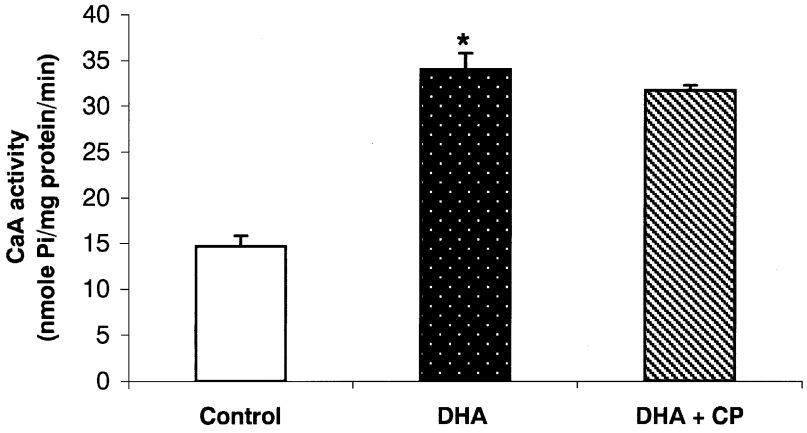

Fig. 3 The effect of DHA and the protein kinase C blocker CP on Ca-ATPase in calmodulin-free SMs. After a 5-min pre-incubation with DHA with or without CP, ATP was added to start the 10 min enzyme reaction. Final concentrations of DHA, CP and SM protein in the reaction mixture were 30 μg/ml, 0.5 μM and 1620 μg/ml, respectively. The addition of CP could not reverse activation by DHA significantly: The mean ± SEM was calculated from a minimum of three independent observations. The data were analysed by one-way ANOVA and *P* calculated by Tukey's test. The activity of DHA-treated samples differed significantly from untreated controls (**P* < 0.05).

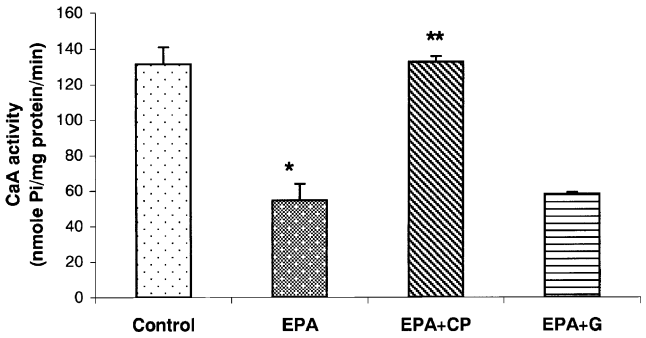

Fig. 4 The influence of the protein kinase C blocker CP and the tyrosine kinase blocker G on EPA inhibition of SM Ca-ATPase activity. After a 5-min pre-incubation of SM with EPA and CP or G, ATP was added to start the 10 min enzyme reaction. Final concentrations of EPA, CP, G and synaptic membrane protein in the reaction mixture were 20 μg/ml, 0.5 μM, 50 μM and 150 μg/ml, respectively. The mean ± SEM was calculated from a minimum of three independent observations. The data were analysed by one-way ANOVA and *P* calculated by Tukey's test. The activity of EPA-treated samples differed significantly from untreated controls (**P* < 0.05). The addition of CP, but not G, could reverse the action of EPA significantly (***P* < 0.05).

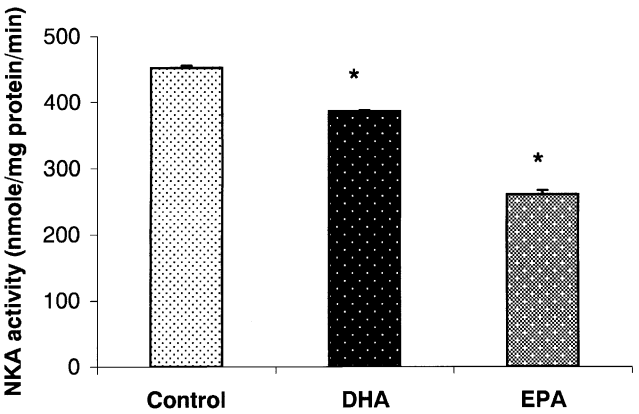

Fig. 5 The effect of DHA and EPA on SM Na,K-ATPase. After a 5-min pre-incubation with DHA or EPA, ATP was added to start the 10 min enzyme reaction. Final concentrations of fatty acids and SM protein in the reaction mixture were 30 μg/ml and 137 μg/ml, respectively. The mean ± SEM was calculated from a minimum of three independent observations. The data were analysed by one-way ANOVA and *P* calculated by Tukey's test. The activity of DHA-treated samples differed significantly from untreated controls (**P* < 0.05).

DHA could activate the enzyme in purified basolateral membranes from rabbit colon,[5] and Ca-ATPase from erythrocytes could be activated by a variety of fatty acids.[12] Different mechanisms of fatty acid action may be involved: direct interactions with Ca-ATPase[11,13] and action on protein kinases A and C[12] that cause ultimate protein phosphorylation of Ca-ATPase are some of the possibilities.

Polyunsaturated fatty acids have been reported to activate[22,23] protein kinase C, depending on the availability of Ca or the specific isoform(s) of protein kinase C in the preparation. In contrast with these early reports, Mirnikjoo et al.[11] has shown that both DHA and EPA have inhibitory effects on protein kinase C. The lack of conclusive evidence on the action of omega-3 fatty acids on Ca-ATPase from brain cortex prompted the current investigation.

This study shows that both DHA and EPA inhibit Ca-ATPase in synaptic membranes. These inhibitory effects could be due to the inhibition of protein kinase C, thus preventing phosphorylation and therefore activation of Ca-ATPase. Our findings show that EPA, but not DHA,

exerts its inhibitory effects on Ca-ATPase via this pathway. DHA may rather exert its effects bound to a phospholipid in the membrane, and not as a free fatty acid, as suggested by Salem et al.[24] Recently, tyrosine kinase also has been shown to be a target for polyunsaturated fatty acid action.[25] However, according to our findings, both DHA and EPA do not exert their inhibitory effects via tyrosine kinase.

Polyunsaturated fatty acids can mimic the activating effect of calmodulin on Ca-ATPase but only when calmodulin is removed from the membrane, as described by Niggli et al.[11] In accordance with these results, this study shows that DHA has an activating effect on Ca-ATPase in calmodulin-stripped synaptosomal membranes. Ca^{2+}-ATPase is activated by DHA by slightly more than 100% when compared to controls, these stimulatory effects are not due to the action of protein kinase C. Calmodulin exerts its stimulatory effects by interaction with the long C terminal domain[13] of the enzyme. Omega-3 fatty acids probably have a direct activating interaction with the S2–S3 loop in the polypeptide chain of Ca-ATPase,[13] thus preventing interaction with calmodulin. Since calmodulin is a truly ubiquitous protein, this type of interaction between fatty acids and Ca-ATPase could only be of physiological importance during very low Ca availability, when the affinity of calmodulin for Ca-ATPase would become minimal.

One particular protein that has been shown to be depressed by omega-3 fatty acids is the voltage-gated calcium channel,[26,27] this would prevent Ca entry into the cell. The findings that DHA and EPA have inhibitory effects on Ca-ATPase and Na,K-ATPase (which drives the Na–Ca exchanger) show that they would cause retention of intracellular Ca. These effects could all contribute to the alleged dampening effect of omega-3 fatty acids on neuronal activity.

Changes in membrane composition by the direct incorporation of omega-3 fatty acids may cause significant conformational changes in the membrane-bound proteins, this may also explain the changes in enzyme activity in the present report. In fact, polyunsaturation in cell membranes has been shown to cause changes in protein kinase C activity.[28] Further research should aim at investigating how changes in membrane composition by omega-3 fatty acids may influence the activity of the plasma membrane Ca-ATPases.

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