

Protein Kinase Inhibition by ω -3 Fatty Acids*

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Recent data suggest that ω -3 fatty acids may be effective in epilepsy, cardiovascular disorders, arthritis, and as mood stabilizers for bipolar disorder; however, the mechanism of action of these compounds is unknown. Based on earlier studies implicating ω -3 fatty acids as inhibitors of protein kinase C activity in intact cells, we hypothesized that ω -3 fatty acids may act through direct inhibition of second messenger-regulated kinases and sought to determine whether the ω -3 double bond might uniquely confer pharmacologic efficacy and potency for fatty acids of this type. In our studies we observed that ω -3 fatty acids inhibited the *in vitro* activities of cAMP-dependent protein kinase, protein kinase C, Ca^{2+} /calmodulin-dependent protein kinase II, and the mitogen-activated protein kinase (MAPK). Our results with a series of long-chain fatty acid structural homologs suggest an important role for the ω -3 double bond in conferring inhibitory efficacy. To assess whether ω -3 fatty acids were capable of inhibiting protein kinases in living neurons, we evaluated their effect on signal transduction pathways in the hippocampus. We found that ω -3 fatty acids could prevent serotonin receptor-induced MAPK activation in hippocampal slice preparations. In addition, we evaluated the effect of ω -3 fatty acids on hippocampal long-term potentiation, a form of synaptic plasticity known to be dependent on protein kinase activation. We observed that ω -3 fatty acids blocked long-term potentiation induction without inhibiting basal synaptic transmission. Overall, our results from both *in vitro* and live cell preparations suggest that inhibition of second messenger-regulated protein kinases is one locus of action of ω -3 fatty acids.

There are twelve essential fatty acids (EFAs)¹ that are subdivided into two structural categories depending on the saturation state of the molecule. The designation omega-3 (ω -3) or omega-6 (ω -6), respectively, indicates if the third or sixth car-

bon from the methyl terminus is unsaturated. The ω -3 and ω -6 fatty acids are derived from the dietary intake of the 18-carbon precursors α -linolenic acid and linoleic acid, respectively. The ω -3 fatty acids include: α -linolenic acid (18:3 ALA), stearidonic acid (18:4), eicosatetraenoic acid (20:4), eicosapentenoic acid (20:5 EPA), docosapentenoic acid (22:5), and docosahexanoic acid (22:6 DHA).

Dietary provision of long-chain ω -3 and ω -6 fatty acids is essential because mammals are incapable of synthesizing fatty acids with a double bond past the Δ -9 position; thus, dietary intake of EFAs has far reaching consequences on membrane composition in all cells in the body and may influence neural function as well. The elucidation of the role for EFAs in neuronal function has followed two disparate lines of investigation. One approach has focused on the effects of dietary intake of EFAs on neuronal membrane composition and function. The reduction of dietary ω -3 fatty acids has been shown to have deleterious effects on cAMP-dependent protein kinase A (PKA) and protein kinase C (PKC) activities (1, 2), brain membrane lipid composition (3), neurotransmission (4, 5), and learning ability in rats (6). In addition, increasing dietary intake of ω -3 fatty acids can improve learning and memory tasks in young rats and overcome deficits in long-term potentiation (LTP, Ref. 7), a robust form of synaptic plasticity, in aged rats. These effects are theorized as being because of physical alterations in membrane characteristics, changing either the fluidity or rigidity of the membrane itself, and affecting neurotransmitter release as well as receptor and channel function (8–10).

An alternative investigative approach has focused on the effect *in vitro* of direct application of ω -3 fatty acids on cellular biochemistry and synaptic transmission. Early reports showed that a variety of fatty acids are capable of activating PKC (11–13); however, in other studies ω -3 and ω -6 fatty acids caused inhibition when PKC was activated with phosphatidylserine (PS) and diacylglycerol (DAG) (14). This latter effect may explain more recent reports that the application of ω -3 fatty acids to hippocampal slices can decrease membrane excitability and block low frequency stimulation-induced long-term depression (LTD, Refs. 15–17). Overall these reports suggest a multifaceted role for ω -3 EFAs involving both direct and indirect actions on neural function and suggest the hypothesis that ω -3 fatty acids may affect protein kinase activity.

Questions concerning the mechanisms of action of EFAs are not trivial, as EFAs have been shown to have profound behavioral effects in humans *in vivo*. For example, dietary EPA and DHA elicit beneficial effects in certain neuropsychiatric disorders, such as bipolar disorder and schizophrenia. New studies of patients with manic-depression have shown significant reduction in reoccurrence of symptoms when the standard lithium treatment is supplemented with ω -3 fatty acids (18). The exact cellular and biochemical mechanisms underlying the mood-stabilizing effect of ω -3 fatty acids are mysterious, mak-

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¹ The abbreviations used are: EFA, essential fatty acid; PKA, cAMP-dependent protein kinase A; PKC, protein kinase C; CaMKII, Ca^{2+} /calmodulin-dependent protein kinase II; MAPK, mitogen-activated protein kinase; HFS, high frequency stimulation; LTP, long-term potentiation; LTD, long-term depression; DHA, docosahexanoic acid; BA, behenic acid; DTEA, *cis*-7,10,13-docosatetraenoic; EPA, eicosapentenoic acid; AA, arachidonic acid; ESEA, *cis*-11,14,17-eicosatrienoic acid; DPEA, *cis*-7,10,13,16,19-docosapentaenoic acid; VPA, valproic acid; 2-ene-VPA, 2-ene-valproate.

ing this a necessary and attractive area for study.

To better understand the biochemical affects of ω -3 fatty acids on the CNS, we focused our attention on their effects on the activities of prevalent protein kinases of the CNS, using kinase assays *in vitro*. We found that DHA and EPA reduced the activity of protein kinase C, cAMP-dependent protein kinase A, mitogen-activated protein kinase (MAPK) (ERK1 and ERK2), and Ca^{2+} /calmodulin-dependent protein kinase II (CaMKII) at low concentrations (IC_{50} 2–36 μM). These effects appear to be dependent on the ω -3 double bond within the fatty acid, as structurally similar compounds saturated at the ω -3 position had little or no effect on kinase activity. We also used the hippocampal slice preparation to assess whether ω -3 fatty acids were capable of affecting protein kinase activity in the living neuron. In one series of studies, we observed that DHA and EPA inhibited serotonin (5-HT) receptor-induced activation of MAPK. In another study, we capitalized on the fact that hippocampal LTP is known to require protein kinase activation. We observed that perfusion of DHA or EPA onto hippocampal slices interfered with the induction of LTP in area CA1, presumably through the reduced activity of multiple protein kinases and disruption of the signaling pathways in which they are involved. Thus, the ω -3 fatty acids are surprisingly potent and efficacious broad-spectrum protein kinase inhibitors, suggesting that protein kinases may be a target of action for ω -3 fatty acids *in vivo*.

MATERIALS AND METHODS

Chemicals— $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was obtained from Amersham Pharmacia Biotech. The catalytic subunit of protein kinase C from rat brain was purchased from CalBiochem-Novabiochem Corp., protein kinase A catalytic subunit from Sigma Chemical Co., Ca^{2+} /calmodulin-dependent protein kinase II from New England BioLabs Inc., and activated MAPK from Stratagene. General laboratory reagents were purchased from commercial sources and were of analytical quality.

Fatty Acids—Docosahexaenoic acid (DHA) [22:6(ω -3)]; behenic acid (BA) [22:0]; *cis*-7,10,13-docosatetraenoic (DTEA) [22:4(ω -6)]; eicosapentaenoic (EPA) [20:5(ω -3)]; arachidic [20:0]; arachidonic [20:4(ω -6)]; *cis*-11,14,17-eicosatrienoic (ESEA) [20:3(ω -3)]; *cis*-7,10,13,16,19-docosapentaenoic (DPEA) [22:5(ω -3)]; and Valproic acids were all obtained from Sigma Chemical Co. 2-ene-valproate was kindly provided by Dr. Wolfgang Loescher and Dr. Danny D. Shen. Free fatty acids were made as 20 mM stock solutions in ethanol (vehicle) and stored under nitrogen, in the dark at -20°C until needed.

Protein Kinase Assays

PKC Assay—Enzyme activity was measured by quantifying incorporation of $[\text{P}^{32}]\text{PO}_4$ into a synthetic peptide substrate, amino acids 28–43 of neurogranin, PKC selectide, CalBiochem. Various concentrations of fatty acids, ranging from 0.4 to 400 μM were incubated with the catalytic domain of protein kinase C from rat brain. The reaction mixtures contained 10 ng of the PKC catalytic domain; reaction buffer (200 mM Tris, 5 mM EGTA, 10 mM EDTA, 20 mM Na_2PO_4); 10 μM of peptide substrate; $[\gamma\text{-}^{32}\text{P}]\text{ATP}$; 0.5 mM ATP and H_2O in a final assay volume of 50 μl . The reaction samples were incubated at 37°C for 20 min and terminated by addition of 25 μl of stop solution (225 mM H_3PO_4 , 1 mM ATP). Two aliquots from each sample were transferred onto Whatman P-81 paper. After a 2-min incubation at room temperature, the filter papers were washed three times for 10 min in 0.25 M H_3PO_4 and one time for 2 min in 95% (v/v) ethanol with gentle agitation. Chromatography papers were air dried prior to quantitation by liquid scintillation counting.

PKA Assay—Assays for PKA activity were performed as described in Roberson and Sweatt (19) with the exception that PKC and CaMKII inhibitors were absent from the reaction buffer, and the chromatography papers were washed in 75 mM H_3PO_4 three times for 10 min before rinsing in methanol. Kemptide (100 μM) was used as the substrate for PKA.

CaMKII Assay—The assays were performed as described in Roberson and Sweatt (19). Reaction conditions consisted of 20 mM Tris-HCl, 10 mM MgCl_2 , 0.5 mM dithiothreitol, 0.1 mM Na_2EDTA , 100 μM autamtide, 25 units of CaMKII, 2.4 μM calmodulin, and 2 mM CaCl_2 (to activate the enzyme), 100 μM ATP and $\gamma\text{-}^{32}\text{P}$ -labeled ATP to a final

specific activity of 100 $\mu\text{Ci}/\mu\text{mol}$. The mixture was incubated for 10 min at 30°C before stop solution was added.

MAPK Assay—Reaction mixtures (prepared on ice) contained 20 ng of (1:5 dilution of 0.1 $\mu\text{g}/\mu\text{l}$ MAPK) activated MAPK, 25 mM HEPES, pH 7.5, 10 mM magnesium acetate, 500 μM ATP; 0.5 $\mu\text{g}/\mu\text{l}$ Phas-I, $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ 1.0 $\mu\text{Ci}/\mu\text{l}$, and various concentrations of DHA and EPA (0.1–100 μM) in a final assay volume of 40 μl . The reaction samples were incubated 30°C for 30 min and terminated by placing the reaction samples on ice with 5 μl of 0.25 M H_3PO_4 (stop solution). Two aliquots of each sample were transferred onto P-51 chromatography paper. After 2 min of incubation at room temperature, the filter papers were washed four times for 3 min each in 100 ml of 75 mM H_3PO_4 and then briefly in 95% (v/v) ethanol with gentle agitation. Chromatography papers were air-dried before the $\gamma\text{-}^{32}\text{P}$ incorporation was quantitated by liquid scintillation counting.

Hippocampal MAPK Activation and Quantitation—Hippocampal slices (400 μm) were prepared from 8–12 week-old male mice as described previously (19). Slices were allowed to recover in artificial cerebral spinal fluid (ACSF: 125 mM NaCl, 2.5 mM KCl, 1.24 mM NaH_2PO_4 , 25 mM NaHCO_3 , 10 mM D-glucose, 2 mM CaCl_2 , and 1 mM MgCl_2) for 3 h at 32°C . ω -3 fatty acids or control fatty acids (10 μM) were added to slices for 1 h prior to 5-HT addition. 5-HT was added to slices at a 10 μM concentration for 10 min at which time the slices were collected and frozen on dry ice. Slices were then homogenized, and samples were prepared as described previously (19). Each *n* represents the pooled protein homogenate from three whole hippocampal slices. The samples were electrophoresed on 10% SDS-polyacrylamide gels, and transferred to Immobilon-P membranes. Membranes were blocked in 5% dry milk solution and 1 μM microcystine for 1 h. All blots were incubated at room temperature and washed in TTBS buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.05% Tween 20). Immunoreactivity was assessed using the enhanced chemiluminescence method (Amersham Pharmacia Biotech). The bands corresponding to phospho-42 MAPK on each Western blot were quantified by densitometry using a StudioScan desktop scanner and NIH Image software.

Hippocampal Slice Physiology—Hippocampal slices (400 μm) were prepared from 8–12 week-old male mice as described previously (19). Slices were perfused (1 ml/min) with ACSF in an interface chamber maintained at 25°C . Field recordings of the Schaffer collateral synapse were monitored for a minimum of 10 min before fatty acid application to ensure a stable baseline. Responses are presented as an average of 6 individual traces. Baseline stimulus intensities were determined from the intensity that produced a field EPSP at 50% of the maximal response. LTP was induced with two trains of 100-Hz stimulation for 1 s, separated by 20 s with the same stimulus intensity used in baseline recordings. Slices were perfused with ACSF at 1 ml/min with either fatty acids diluted to 10 μM or ACSF with the addition of an equal volume of vehicle. Fatty acids were perfused onto the slice 20 min prior to HFS and remained for 20 min after HFS delivery for a total of 40 min (see Fig. 7; B–D, black bar).

Data Analysis—The GraphPad Prism and Microsoft Power Point software packages were used for curve-fitting and statistical analyses.

RESULTS

The therapeutically beneficial ω -3 fatty acids (typically found in fish oils) are long-chain, unbranched molecules. In the present studies we used a series of structurally related long-chain fatty acids to investigate the structure/function relationships for this category of compounds, examining their efficacy as inhibitors of protein kinases (Fig. 1). For the DHA family of fatty acids we investigated three structurally similar compounds. Docosahexaenoic acid [22:6 (ω -3), DHA] is a twenty-two carbon ω -3 fatty acid with a total of six double bonds. Compounds used as DHA controls included Behenic acid [22:0, BA], the saturated form of DHA lacking all double bonds including the ω -3 double bond, and *cis*-7,10,13,16-docosatetraenoic acid [22:4 (ω -6), DTEA], which lacks the ω -3 double bond and the number four double bond (Fig. 1). Eicosapentaenoic acid [20:5 (ω -3), EPA] is a twenty carbon ω -3 fatty acid with a total of five double bonds. Arachidic acid [20:0], the saturated form of EPA, and Arachidonic acid [20:4 (ω -6), AA], lacking the ω -3 double bond, were also investigated as EPA controls (Fig. 1).

DHA and EPA Inhibit the PKA Catalytic Subunit—We first

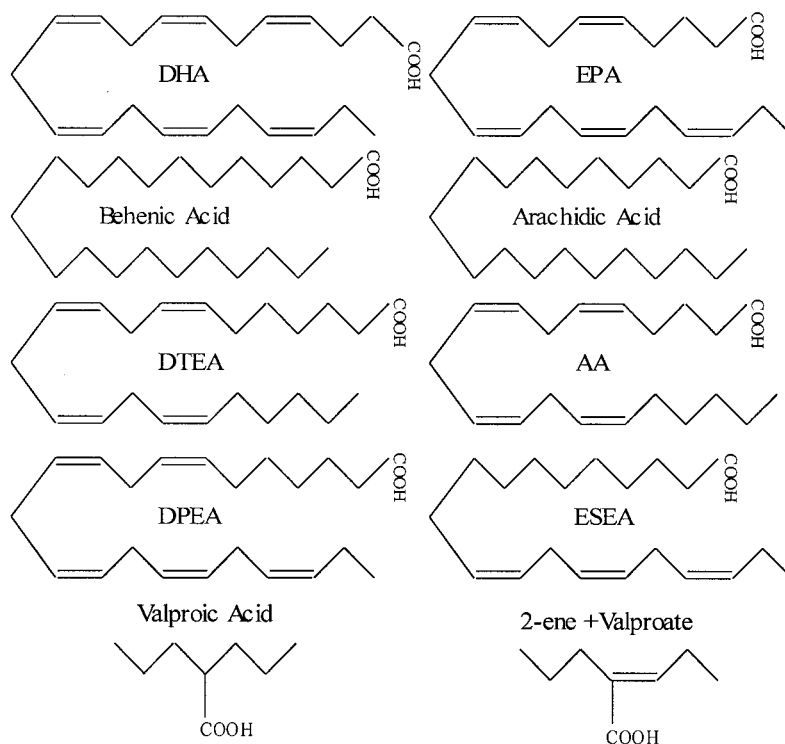


FIG. 1. **Structures of ω -3 fatty acids and control isomers.** A graphic representation of the fatty acids used in these experiments is shown. DHA, docosahexaenoic acid [22:6 (ω -3)]; Behenic acid (saturated DHA) [22:0]; DTEA, *cis*-7,10,13,16-docosatetraenoic acid (DHA analog lacking the ω -3 double bond) [22:4 (ω -6)]; DPEA, *cis*-7,10,13,16,19-docosapentaenoic acid [22:5 (ω -3)]; EPA, eicosapentaenoic acid [20:5 (ω -3)]; arachidic acid (the saturated form of EPA) [20:0]; AA, arachidonic acid (EPA isomer lacking the ω -3 double bond) [20:4 (ω -6)]; ESEA, *cis*-11,14,17-eicosatrienoic acid; valproic acid [8:0]; 2-ene-valproate [8:1 ω -3].

tested if the parent ω -3 compounds DHA and EPA caused an effect on PKA catalytic subunit activity. To determine the potency of DHA and EPA as PKA inhibitors, we used various concentrations of fatty acids (0.4–400 μ M) in assays *in vitro* for PKA phosphorylation of Kemptide substrate. Both DHA and EPA significantly inhibited the activity of the PKA catalytic subunit with an IC_{50} of 34 μ M for DHA and 2 μ M for EPA (Fig. 2, A–B). Compounds such as BA and DTEA (Fig. 1) lacking the ω -3 moiety were used as controls for DHA. BA, the saturated form of DHA, did not significantly inhibit PKA activity (Fig. 2A). DTEA, which not only lacks the ω -3 double bond but also lacks the double bond in the fourth position, had a substantially higher IC_{50} than DHA, with an IC_{50} of 230 μ M. These results demonstrate that DHA is more potent in PKA inhibition than BA and DTEA and suggest that the ω -3 double bond is important in conferring potency and efficacy for inhibition of PKA. As controls for EPA we evaluated arachidic acid and AA. Arachidic acid, the saturated form of EPA lacking the ω -3 double bond, was not as potent in the inhibition of PKA phosphotransferase activity as EPA. This was evident in the calculated IC_{50} of >400 μ M for arachidic acid. In addition, a calculated IC_{50} of 59 μ M for AA, which is structurally equivalent to EPA except for the lack of the ω -3 double bond, was 30-fold greater than the IC_{50} for EPA of 2 μ M (Fig. 2B). Overall these data indicate that both DHA and EPA effectively inhibit PKA activity *in vitro* and suggest that the ω -3 double bond plays an important role in conferring inhibitory efficacy and potency for both types of compounds.

The ω -3 Fatty Acid Moiety Is Important in Inhibiting PKA Activity—Given the results described above, we wanted to test if the presence of the ω -3 double bond was sufficient to confer inhibitory efficacy. We therefore tested two other long-chain fatty acids that have the ω -3 double bond moiety incorporated into their structure. Toward this end we evaluated *cis*-11,14,17-eicosatrienoic acid (ESEA), a twenty-carbon ω -3 fatty

acid with three double bonds, and *cis*-7,10,13,16,19-docosapentaenoic acid (DPEA), a twenty-two carbon ω -3 fatty acid with a total of five double bonds (Fig. 1). We examined whether ESEA or DPEA exhibited inhibitory effects by evaluating the effect of increasing concentrations (1.0–400 μ M for ESEA and 0.1–400 μ M for DPEA) on PKA phosphotransferase activity (Fig. 2C). We found that both ESEA and DPEA blocked PKA catalytic subunit activity; IC_{50} values were calculated to be 22 μ M for ESEA and 10 μ M for DPEA. Once again these data suggest that the ω -3 double bond plays an important role in the inhibition of PKA by these long-chain fatty acids. This is illustrated quite nicely, for example by comparing the potencies of DTEA *versus* DPEA (Fig. 2, A *versus* C). These two compounds are structurally identical with the exception of the ω -3 double bond contained within DPEA, which showed much greater potency than DTEA in PKA inhibition.

EPA Is Not a Competitive Inhibitor of ATP—Most broad-spectrum inhibitors of second messenger-regulated kinases are competitive with ATP (20, 21), and we sought to determine whether this was the mechanism of action of ω -3 fatty acids. If ω -3 fatty acids are competitive with ATP, then higher ATP concentrations should cause a shift to a higher IC_{50} for ω -3 fatty acid inhibition of PKA. We therefore compared the IC_{50} for EPA inhibition of PKA, using two different final ATP concentrations: 100 and 500 μ M. EPA reduced PKA activity indistinguishably under both concentrations with no significant difference in inhibition of PKA activity (IC_{50} for both ATP concentrations was \sim 2 μ M, Fig. 3). These data strongly suggest that EPA is not a competitive inhibitor of ATP binding to PKA.

ω -3 Fatty Acid Inhibition of PKC, CaMKII, and MAPK—If fatty acids unsaturated in the ω -3 position have the capability of inhibiting the catalytic subunit of PKA, then it is reasonable to hypothesize that this effect may transcend to other protein kinases with homologous catalytic domains. Therefore we examined the effect of different long-chain fatty acids on two

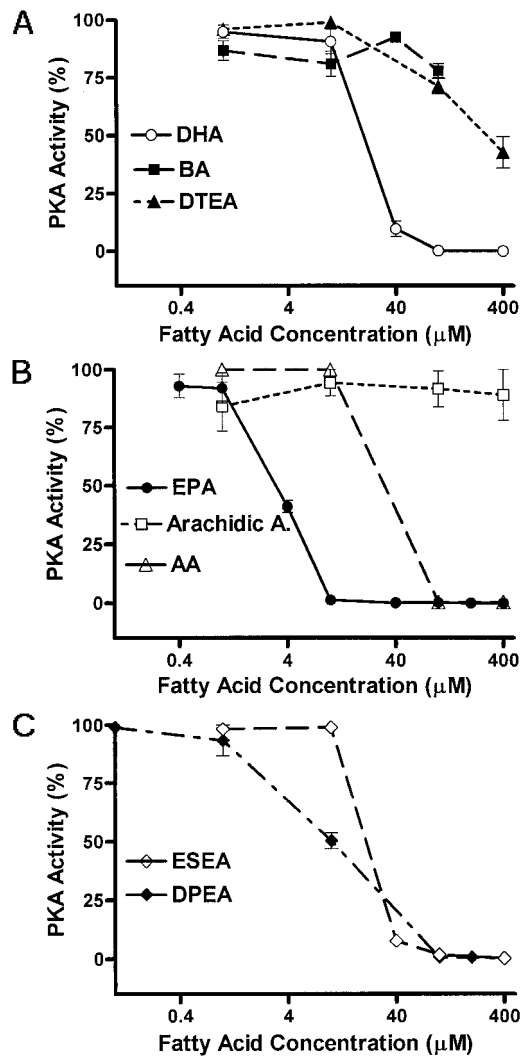


FIG. 2. **Inhibition of PKA by DHA and EPA.** *In vitro* measurement of PKA catalytic domain activity using Kemptide as a substrate. A, DHA ω -3 fatty acid at increasing concentrations (1–400 μ M) inhibits PKA catalytic subunit activity. Behenic acid (BA) and DTEA were used as 22-carbon chain-length control fatty acids. The IC_{50} for DHA is 34 μ M; for BA >400 μ M, and for DTEA, 230 μ M. B, EPA ω -3 fatty acid at increasing concentrations (1–400 μ M) to inhibit PKA catalytic subunit activity. Arachidic acid and AA were used as 20-carbon chain-length control fatty acids. The IC_{50} for EPA is 2.1 μ M; for arachidic acid >400 μ M; and for AA, 59 μ M. C, ESEA and DPEA fatty acids (concentrations of 1–400 μ M and 0.1–400 μ M, respectively) inhibited PKA catalytic subunit activity. The IC_{50} of ESEA is 22 μ M and DPEA is 10 μ M. The results shown are mean \pm S.E. for three experiments in all cases.

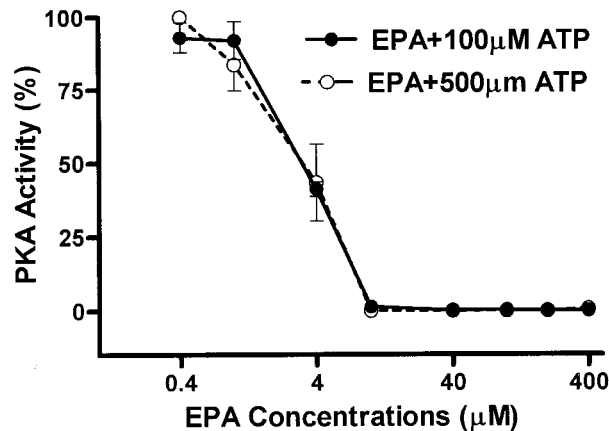


FIG. 3. **EPA is not a competitive inhibitor of ATP.** Concentrations of 100 and 500 μ M ATP were added to the reaction assay to determine whether EPA inhibition of PKA was caused by competition with ATP. The IC_{50} for EPA with 100 μ M ATP is 2.1 μ M and EPA with 500 μ M ATP is 1.7 μ M. The results shown are mean \pm S.E. for three experiments in both cases.

other kinases; PKC and CaMKII. We tested EPA, DHA, and AA on PKC catalytic domain phosphotransferase activity using a peptide substrate (*in vitro*) using increasing fatty acid concen-

trations (Fig. 4A). We found that ω -3 fatty acids inhibited PKC and, similar to results for PKA, EPA was found to be a more potent inhibitor of PKC activity than DHA (IC_{50} for EPA was 2

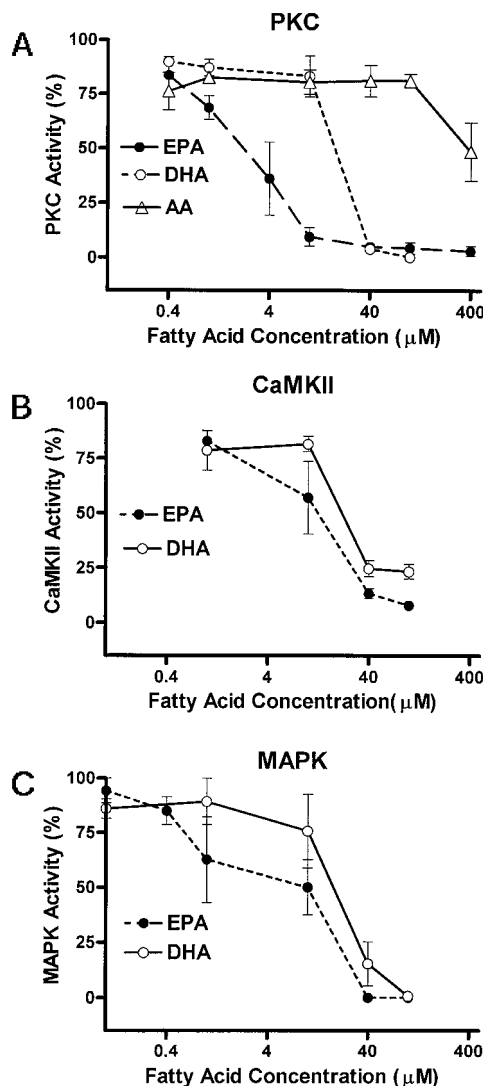


FIG. 4. EPA and DHA inhibition of PKC, MAPK, and CaMKII. A, EPA and DHA inhibit PKC catalytic domain activity. PKC activity was measured as described under "Materials and Methods." The IC_{50} for EPA is 1.8μ M, DHA is 36μ M, and AA $>400 \mu$ M. AA inhibits PKC activity only at high concentrations (400μ M). EPA inhibits the PKC activity at lower concentrations than DHA. B, EPA and DHA inhibition of CaMKII catalytic domain. Measurement of CaMKII activity using autocamtide as a substrate was as described under "Materials and Methods." The IC_{50} for EPA is 15μ M and DHA is 36μ M. EPA inhibits CaMKII activity at a lower concentration than DHA. C, EPA and DHA inhibition of MAPK activity. EPA and DHA concentrations ranged from 0.1 to 100μ M. Results shown are mean \pm S.E. for three experiments in all cases. Measurement of MAPK activity using Phas-1 as a substrate was as described under "Materials and Methods." The IC_{50} for EPA is 23μ M and DHA is 34μ M. Both EPA and DHA inhibit MAPK completely at concentrations higher than 40μ M, but EPA is a more potent inhibitor than DHA.

μ M compared with 36μ M for DHA). AA, which lacks the ω -3 double bond, was used as a control and did not cause significant inhibition of PKC enzymatic activity ($IC_{50} >400 \mu$ M). Similarly, the inhibitory effect of EPA and DHA was also shown in assays using the catalytic subunit of CaMKII (Fig. 4B). Both EPA and DHA inhibited CaMKII activity at relatively low concentrations with a calculated IC_{50} for EPA of 15μ M and for DHA of 36μ M. Thus similar to what was observed with PKC and PKA, EPA and DHA inhibit CaMKII.

The second messenger-regulated kinases PKA, PKC and CaMKII share sequence homology within their catalytic domains. The ERK MAPKs are regulated by phosphorylation and not second messengers, but nevertheless exhibit structural homology to the PKA, PKC, and CaMKII catalytic domains (22, 23). We therefore determined if ω -3 fatty acids inhibited MAPK activity. To determine the potency of EPA and DHA at inhibiting MAPK activity, increasing concentrations of both fatty acids (0.1 – 100μ M) were used (Fig. 4C). Both ω -3 fatty acids inhibited MAPK activity, and EPA was a slightly more potent inhibitor of MAPK activity than DHA (IC_{50} for EPA was 23μ M

and for DHA, 34μ M). Overall these data show that DHA and EPA act as broad-spectrum kinase inhibitors, blocking not only the activity of PKA, PKC, and CaMKII, but also the activity of MAPK.

VPA and 2-ene-VPA Exhibit No Effect on PKC or PKA—Valproic acid (VPA, 2-propylpentanoic acid), is a short chain length, eight carbon, branched fatty acid (Fig. 1) and, like ω -3 fatty acids, VPA has recently been used for treatment of bipolar disorder (24, 25). 2-ene-VPA (2-ene-valproate, 2-propyl-2-pentenoic acid, Fig. 1) is the major VPA metabolite in humans, which also contains the ω -3 moiety. Given that VPA and the ω -3 fatty acids DHA and EPA exhibit similar neurologic effects, we thought it an intriguing possibility that 2-ene-VPA might inhibit protein kinases in a fashion similar to the long-chain fatty acids described above. Therefore we tested whether VPA or 2-ene-VPA had the same inhibitory effect as long-chain ω -3 fatty acids on PKA and PKC activity *in vitro*. As expected, we found a lack of an inhibitory effect of VPA on PKC or PKA phosphotransferase activity at concentrations ranging from 1 to 200μ M (data not shown). Interestingly, there was also a lack

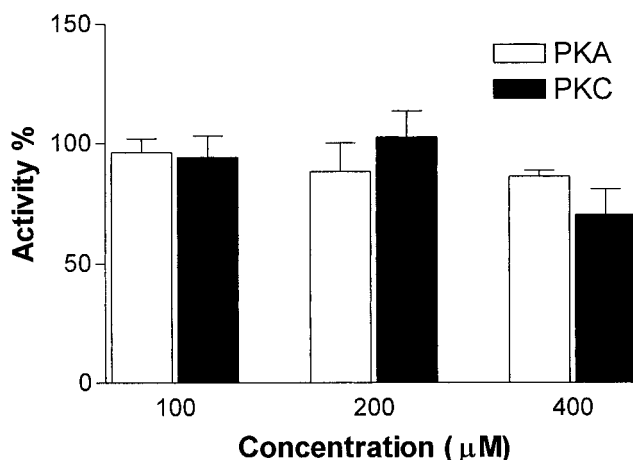


FIG. 5. **Lack of effect of 2-ene-VPA on PKC and PKA activity.** Data are presented as the percent change of PKA (\square) and PKC (\blacksquare) catalytic domain activity in the presence of 2-ene-VPA compared with control assays. The *in vitro* action of 2-ene-VPA on PKA and PKC activities were determined using increasing concentrations of 100, 200, and 400 μ M. Results shown are mean \pm S.E. for $n = 3$.

of inhibition of PKA and PKC activity with 2-ene-VPA using concentrations of 100, 200, and 400 μ M (Fig. 5). Thus, the ω -3 moiety within the 2-ene-VPA does not confer significant inhibitory efficacy for PKC or PKA activity *in vitro*. This lack of effect of 2-ene-VPA may be because of its overall short chain length or the proximity of the carboxylic acid in relation to the ω -3 double bond. These data also indicate that neither VPA nor its major metabolite act by directly inhibiting PKA or PKC, suggesting that a different mechanism of action exists *in vivo* for VPA versus ω -3 long-chain fatty acids.

DHA and EPA Can Inhibit Serotonin-induced Activation of MAPK—Whereas the results thus far demonstrate that DHA and EPA have effects on kinase activity *in vitro*, they do not address whether the compounds are capable of inhibiting protein kinases in the intact cell. For the next stage of our studies we sought to develop a preparation that we could use to test the efficacy of ω -3 fatty acids in inhibiting protein kinases in living cells. The serotonergic signal transduction pathway can activate MAPK in a PKA-dependent process in aplysia sensory neurons, but the effects of 5-HT on MAPK in the mammalian brain have not been determined (26). We reasoned that investigating serotonin-coupled kinase activation was a desirable avenue of pursuit because the serotonergic system is widely implicated in affective disorders for which ω -3 fatty acids have beneficial effects. In our first studies, we found that 10 μ M 5-HT caused MAPK activation in hippocampal slices evaluated by the increased phosphorylation of p42 MAPK (Fig. 6), an effect blocked by EPA and DHA. The control compounds behenic acid and arachidic acid showed no inhibition of 5-HT receptor-induced MAPK activation. Application of ω -3 fatty acids (DHA and EPA) or control fatty acids (behenic acid and arachidic acid) to hippocampal slices alone had no effect on basal MAPK activation (data not shown). As an additional control we used the MEK inhibitor U0126 on 5-HT receptor-induced Erk activation. The compound U0126 completely blocked 5-HT-induced activation of MAPK in the hippocampus. Taken together, these results suggest that ω -3 fatty acids are capable of blocking serotonin-induced kinase activation in living neurons in the hippocampus.

DHA and EPA Inhibit Induction of Hippocampal LTP in Area CA1—The very kinases shown here to be inhibited by ω -3 fatty acids have been shown previously to be necessary for mammalian synaptic plasticity, specifically LTP in the hippocampus. To determine the effect of ω -3 fatty acids on this kind of kinase-dependent synaptic plasticity in intact living neurons, we tested the ability of EPA and DHA to block the

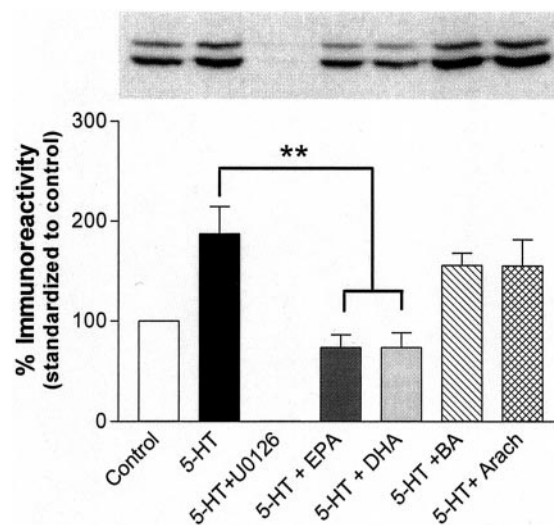


FIG. 6. **Effect of EPA and DHA on serotonergic signal transduction.** Serotonin activation of MAPK can be blocked by DHA and EPA, but not behenic acid or arachidic acid. Effect of a 10-min application of 5-HT on MAPK activation in mouse hippocampal slices (lane 2) compared with 5-HT activation following a 1-h incubation with DHA, EPA, behenic acid (BA), and arachidic acid (lanes 4–8, respectively). Controls consisted of untreated (lane 1) and slices treated with the MEK inhibitor U0126 (lane 3). *Top*, representative immunoblot for phospho-p42 and phospho-p44 MAPK from whole hippocampal homogenates. Bands correspond to the labeled column directly beneath. *Bottom*, quantitative Western analysis of immunoblots that were probed for phospho-MAPK (p42) and standardized to nontreated controls. Application of ω -3 fatty acids (DHA and EPA) or control fatty acids (behenic acid and arachidic acid) to hippocampal slices alone had no effect on basal MAPK activation (data not shown). Columns and error bars represent the mean \pm S.E. from $n = 3$ or 5 Western blot analyses (**, $p < 0.01$).

induction of LTP in area CA1 of the hippocampus. The application of HFS (two trains at 100 Hz for 1 s, separated by 20 s) is sufficient to induce a long lasting potentiation at Schaffer collateral synapses (Fig. 7A). Application of DHA (10 μ M) did not affect baseline synaptic transmission; however, perfusion prior to HFS completely blocks the induction of LTP (Fig. 7C). Like DHA, EPA has little effect on baseline synaptic transmission and also blocks induction of LTP (Fig. 7D). As a control, we perfused slices with the long-chain fatty acid AA prior to HFS. (Fig. 7B). Slices treated with AA showed potentiation (>150% compared with baseline) that was not different from untreated slices. Our results are consistent with the observations of

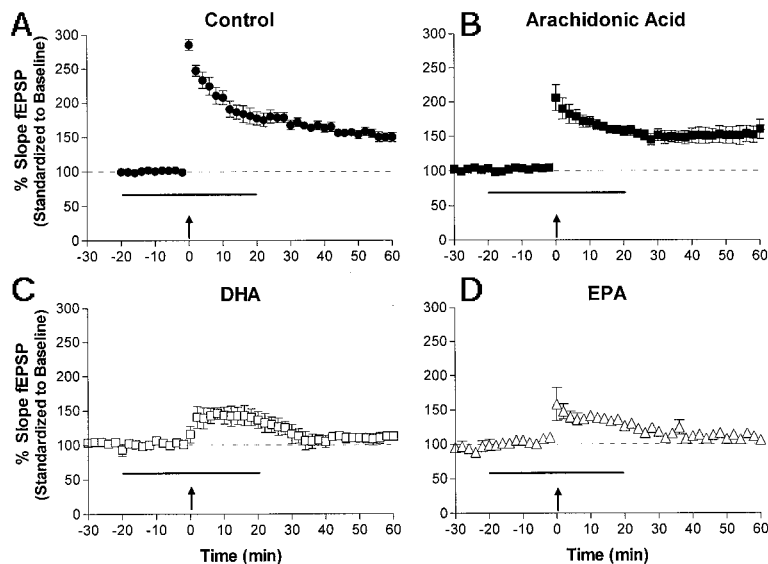


FIG. 7. **Effect of DHA and EPA on Hippocampal LTP in area CA1.** LTP in area CA1 of the hippocampus can be induced by HFS consisting of two trains of 100-Hz stimulation for 1 s separated by a 20-s interval (indicated by an arrow). In control slices, a stable 20-min baseline recording was followed by HFS resulting in a long-lasting increase in synaptic potentiation (A). Following a stable 20-min baseline recording, vehicle (ETOH)(A), AA (B), DHA (C), or EPA (D) ($10 \mu\text{M}$) was bath-applied (black bar) for 20 min prior to HFS and maintained for another 20 min following HFS (40 min total). All recordings are normalized to the mean field excitatory postsynaptic potential (fEPSP) of the initial 20-min baseline recordings. Data points and error bars represent the mean \pm S.E. from $n = 4$ slice recordings.

Itokazu *et al.* (27), who show that intracerebroventricular injection of DHA inhibited the induction of LTP in hippocampal area CA1, *in vivo*. Our studies examining the effect of the ω -3 moiety show that both EPA and DHA can disrupt LTP in hippocampal slice preparations and that LTP is unaffected with the structural analog AA that lacks the ω -3 double bond. Interestingly, the effects of DHA and EPA on tetanus-induced potentiation in hippocampal area CA1 are typical of the effect of broad-spectrum kinase inhibitors; blocking late stages of LTP whereas leaving a transient potentiation intact. Overall, our studies suggest that DHA and EPA inhibit LTP induction via the reduction of neural protein kinase activity.

DISCUSSION

The purpose of these studies was to investigate the mechanism of action of ω -3 fatty acids and to clarify the effects of ω -3 fatty acids on specific protein kinases established as playing important roles in signal transduction, synaptic function and plasticity in the mammalian CNS. In our *in vitro* experiments, EPA and DHA significantly reduced the activity of PKA, PKC, MAPK, and CaMKII. This effect was in contrast to control fatty acids of similar composition that were saturated at the ω -3 carbon (BA, DTEA, AA, arachidic acid), which exhibited a greatly diminished potency in kinase inhibition. Our *in vitro* observations that ω -3 fatty acids may act as nonspecific protein kinase inhibitors is an important first step in understanding the potential molecular targets of free ω -3 fatty acids in the cell. To test our hypothesis that ω -3 fatty acid effects on protein kinases can have a physiologic effect in living tissue, we evaluated DHA and EPA effects on mouse hippocampal slices. We found that application of low concentrations of ω -3 fatty acids blocked 5-HT-receptor induced kinase activation and blocked the induction of LTP in area CA1 of the hippocampus. This indicates that ω -3 fatty acids block protein kinase activity in the intact cell as well as *in vitro*, and suggests the interesting possibility that neuronal kinase inhibition may underlie the known therapeutic effects of ω -3 fatty acids in affective disorders.

In our experiments, we measured the phosphotransferase activity of PKA, PKC, MAPK, and CaMKII in the presence of the ω -3 fatty acids DHA and EPA and fatty acids of equal

length with different degrees and sites of saturation. Both DHA and EPA appear to reduce kinase activity at low concentrations *in vitro* with EPA appearing to be slightly more efficient in reducing kinase activity than DHA. Importantly, only the catalytic domains of PKA, PKC and CaMKII were used in these assays. Thus in our experiments ω -3 fatty acid interactions occur at site(s) other than the second messenger binding sites present in the holoenzymes. However, the observed effect of ω -3 fatty acids did not appear to be due to competitive inhibition at the ATP binding domain, as increasing the concentration of ATP in the kinase assay had no effect on the efficacy of EPA inhibition. Recently, Radomska-Pandya *et al.* (28) reported that direct binding of all-*trans*-retinoic acid (atRA) to PKC can significantly reduce its activity *in vitro*; however the enzyme region responsible for this interaction has yet to be identified. Given that atRA is also a long-chain unbranched fatty acid, it is an appealing speculation that atRA and ω -3 fatty acids share a similar locus of action.

We show that addition of 5-HT ($10 \mu\text{M}$) to hippocampal slices can activate MAPK. This activation can be blocked by ω -3 fatty acids, but not with structurally similar fatty acids lacking the ω -3 double bond moiety. Our results showing the effect of ω -3 fatty acids on 5-HT-dependent signal transduction raises the interesting idea that this pathway may be a locus for the mood-stabilizing effects of ω -3 fatty acids. This hypothesis is supported by observations that alterations in serotonergic neurotransmission have been implicated in the pathophysiology of major depression and suicide. Moreover, treatment of depression often involves the regulation of 5-HT-coupled intracellular signal transduction pathways through the therapeutic action of 5-HT specific reuptake inhibitors, which act to desensitize 5-HT receptors and depress serotonergic processes. A normal function for ω -3 fatty acids in serotonergic function may exist, in light of studies showing that chronic ω -3 fatty acid deficiency in rats can alter serotonergic neurotransmission (3), although it is unknown if these effects are because of changes in membrane-associated ω -3 fatty acids or the availability of free ω -3 fatty acids. Overall, our results support the hypothesis that a means by which ω -3 fatty acids may exert their beneficial mood stabilizing actions is through the suppression of 5-HT-depend-

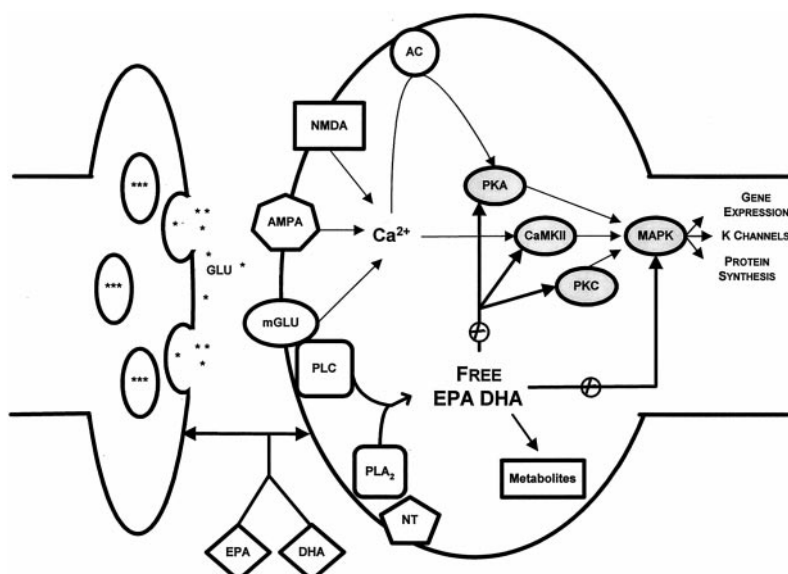


FIG. 8. **A model for EPA and DHA action on synaptic function.** This diagram depicts the possible actions of ω -3 fatty acids within the CNS. DHA and EPA may play a role in at least two cellular areas; the incorporation into the plasma membrane and the presence of intracellular free ω -3 fatty acids. The integration of ω -3 fatty acids may alter membrane dynamics, which subsequently can affect function of postsynaptic receptors and membrane-bound enzymes, as well as presynaptic glutamate release. Free cytoplasmic EPA and DHA in the postsynaptic neuron may exert sufficient inhibitory action on protein kinase activities to alter synaptic plasticity events, dependent on gene expression and protein synthesis, or reduce overall neuronal excitability by blocking modulation of potassium channels.

ent signal transduction pathways.

We determined that perfusion of ω -3 fatty acids prevents the induction of LTP in area CA1 of the hippocampus. The necessity for PKA, PKC, MAPK, and CaMKII in synaptic plasticity is well established, as numerous studies have shown that inhibition of one or more of these kinases, or pathways in which these kinases are involved, will disrupt LTP induction (19, 29–31). Perfusion of hippocampal slices with low concentrations (10 μ M) DHA or EPA, but not arachidonic acid, was sufficient to disrupt induction of LTP. This strongly suggests that the inhibition of CA1 LTP is the result of DHA and EPA-dependent reductions in the activities of several kinases involved in LTP induction. These results may give a glimpse into the ability of ω -3 fatty acids to exert an effect on neuronal function *in vivo*. The ω -3 fatty acid concentrations we used in our LTP studies caused only a modest kinase inhibition in our *in vitro* studies, but were nonetheless able to block LTP induction. It is possible that in these experiments hippocampal slices are concentrating the highly hydrophobic fatty acids, increasing the concentration locally. An interesting alternative possibility is that the effect is because of the kinase-dependent signal integration and amplification that occurs during high frequency stimulus-induced LTP induction. In this scenario, slight reductions in the activity of CaMKII, PKA, PKC, and MAPK sum up to prevent the cell from reaching an LTP induction threshold. Regardless, reducing kinase activity through ω -3 fatty acid application does not appear to interrupt basic synaptic function because the application of EPA or DHA appears to have no effect on normal CA1 synaptic transmission.

The present studies complement previous research into the action of dietary ω -3 fatty acids, specifically on membrane composition, and the associated changes in synaptic function. Work by McGahon *et al.* (5) has shown that increasing the dietary consumption of ω -3 fatty acids has the ability to overcome age-related impairments in LTP. This raises the interesting question of how dietary intake of ω -3 fatty acids can rescue hippocampal LTP in light of studies by Young *et al.* (16) showing that DHA can inhibit low frequency-induced LTD and our studies showing that perfusion of ω -3 fatty acids can disrupt high frequency-induced LTP. It may be that the depletion of

ω -3 fatty acids, through dietary restriction or normal aging, has a deleterious effect on CNS membranes, and thus the supplementation of dietary fatty acids restores synaptic membrane function. However, available data suggest that the idea that ω -3 fatty acids have a role limited to maintaining membrane fluidity is unlikely. Actions of free, unesterified DHA and EPA are suggested in response to a variety of stimuli such as cortical injury (32), modulation of calcium signaling in vascular smooth muscle (33) and in proper retinal function (34, 35). In addition, it has long been known that release of ω -3 fatty acids can occur following electroconvulsive shock and ischemia (36, 37) and, more recently, the identification of specific sites for ω -3 fatty acid accumulation at the synapse (38, 39) would appear to support the hypothesis that both free and membrane-bound ω -3 fatty acids may play diverse roles in synaptic function.

Whereas we have focused our studies on direct effects of ω -3 fatty acids on protein kinases, it is possible, even likely, that ω -3 fatty acids play multiple roles in neuronal function and regulation through direct action upon membrane-bound proteins, alterations in physical membrane characteristics, and direct action upon protein kinases. However, we find it interesting to consider the possible role for direct ω -3 fatty acid-induced inhibition of protein kinases in the CNS. Particularly intriguing is the possibility of stimulus-provoked release of free fatty acids as a mechanism for neuromodulation (Fig. 8). Specifically, we propose that liberation of free ω -3 fatty acids through the receptor-mediated activation of phospholipases may lead to transient local elevation of DHA and EPA and consequent inhibition of protein kinase activity. This biochemical mechanism could serve as a means whereby one receptor could dampen the effect of another simultaneously activated receptor. This raises the possibility that the mood stabilizing effects of increased dietary EPA and DHA resides in the increased abundance of ω -3 fatty acids available for lipase-mediated release (40). The fact that EPA concentrations in the brain are extremely low (<1.0% of total fatty acid, Refs. 41, 42) coupled with our results showing the potent inhibitory effect of very low concentrations of EPA, would support this model.

In conclusion, our results illustrate that one mechanism by which ω -3 fatty acids can affect cellular function is to reduce

the activity of several protein kinases and thereby down-regulate the signal transduction pathways in which these kinases are involved. This potential mechanism should be kept in mind when considering the therapeutic benefits of ω -3 fatty acids in heart disease, inflammation, metabolic disorders, cancer, and neuropsychiatric disorders.

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