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Effects of dietary fish oil on calcium homeostasis in rat platelets

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Mechanisms to explain the differential effects of dietary n-6 and n-3 polyunsaturated fatty acids (PUFA) on cellular functions are not completely understood. In this study, we investigated the effects of these two types of PUFA on ionized calcium (Ca^{2+}) fluxes in platelets. Adult male rats were fed either an n-6 PUFA-rich diet (corn oil, CO) or an n-3 PUFA-rich diet (menhaden oil, MO) for 8 to 12 days. Receptor-stimulated release and basal membrane fluxes of Ca^{2+} were studied in platelets using two different reporter molecules. The fluorescent indicator chlorotetracycline (CTC) was employed to investigate Ca^{2+} mobilization from internal storage compartments upon stimulation. Aequorin, a photoprotein, was used to monitor calcium movements through platelet membranes. Platelets with n-3 PUFA in membrane lipids as a consequence of the MO diet demonstrated a delay in the mobilization of Ca^{2+} from intracellular stores in response to stimulation to several doses of thrombin compared with control platelets (CO). This delayed response was significantly different compared with that found for controls at the lowest dose of agonist (MO, 40.2 ± 3.2 sec; CO, 28.5 ± 1.9 sec, mean ± SEM, P = 0.02). Decay rates for aequorin in rat platelets were found to be linear for 30 min and were similar after exposure to either 0.75 or 1.5 mM external calcium. The rates in platelets from animals fed MO were significantly lower compared with those observed in platelets from the animals fed CO (P = 0.003). The combined results with the reporter molecules suggest that incorporation of n-3 PUFA into membranes delays calcium release and flux under stimulated and basal conditions, respectively. Such alterations would contribute to changes in cellular responses and suggest additional actions of n-3 PUFA beyond those attributed to their inhibitory actions on eicosanoid production from n-6 PUFA. (J. Nutr. Biochem. 6:327–333, 1995.)

Keywords: chlortetracycline; aequorin; calcium signals; omega-6 fatty acids; omega-3 fatty acids; n-3 fatty acids

Introduction

The function of platelets is significantly influenced by dietary fatty acids and the evidence suggests that diets high in n-3 polyunsaturated fatty acids (PUFA) reduce thrombotic risk.[1,2] Dietary modification of platelet phospholipids by replacing n-6 PUFA with n-3 PUFA results in reduced thromboxane A_2 production[3,4] and the ability of platelets to aggregate.[5,6] Thromboxane A_2, a derivative of arachidonic acid, is synthesized upon platelet activation and is a potent amplifier of aggregation. Another important signal generated in platelets during activation is the increase in intracellular ionized calcium. In response to thrombotic stimuli, an increase in the concentration of cytosolic ionized calcium is observed and this increase is associated with a variety of platelet functions such as shape change, aggregation, and secretion.[7] Both intracellular stores and the extracellular media can furnish the ionized calcium needed. Liberation of ionized calcium from intracellular storage compartments is accomplished by second messengers generated during occupancy of cell surface receptors. The influx through receptor-operated channels is also a major contributor to increases in Ca^{2+}.[8] The purpose of this study was to compare the effects of dietary n-6 and n-3 PUFA on both stimulated and basal calcium movements in platelets. Various reporter molecules can be used to investigate such activities in cells. In our experiments we employed two probes, chlortetracycline (CTC) and aequorin, to investigate calcium movements. The fluorescent probe, CTC, was employed to fol-

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low calcium release from internal membrane compartments in thrombin-stimulated platelets[9]; and aequorin, by measuring the decay of luminescence in resting platelets, was used to monitor calcium ion fluxes in the basal state.[10]

Materials and methods

Materials

Collagen was obtained from Chrono-log Corp. (Havertown, PA USA), prostaglandin I_2 (PGI_2) sodium salt was purchased from the Cayman Chemical Co. (Ann Arbor, MI USA), and aequorin was obtained from Dr. John Blinks (Mayo Clinic, Rochester, MN USA). The following reagents were purchased from the Sigma Chemical Co. (St. Louis, MO USA): Sepharose 2B, HEPES (N-(2-hydroxyethyl)piperazine-N′-[(2-ethanesulfonic acid), bovine serum albumin EGTA (ethylene glycol-bis](β-aminoethyl ether)N,N,N′,N′-tetraacetic acid, free acid), dimethyl sulfoxide (DMSO), thrombin (bovine, plasma), sodium pyruvate, β-NADH, reduced form disodium salt, and CTC. Other basic reagents were purchased from the Sigma Chemical Company or from Fisher Scientific (Pittsburgh, PA USA) and were of reagent grade.

Animals and diets

Purified diets (Teklad, Madison, WI USA) were fed to adult male Wistar rats (Hilltop Labs, Inc., Scottdale, PA USA) for 8 to 12 days. The basal diet plus the 5% by weight lipid supplement contained all known requirements for rats. The lipid added was either 5% corn oil (CO) or 4% menhaden oil (Zapata Haynie Corp., Reedville, VA USA) plus 1% corn oil (MO). The animals were housed in a room that was maintained at 25°C with approximately 50% relative humidity. A 12 hr light/dark cycle was employed. The rats were kept in stainless steel cages and given food and water ad libitum. Fresh food was supplied daily. The animals were weighed at the beginning of the diet, several interim times, and at the end of the experiment. There were no differences in the growth rates of the animals between diets. The experimental protocol was reviewed and approved by the Beltsville Area Animal Care and Use Committee.

Blood collection and platelet-rich plasma preparation

Animals were anesthetized with diethyl ether, and blood samples were collected from the heart using an 18 G needle and plastic syringe (3.8% sodium citrate, 1/10 vol). The platelet-rich plasma (PRP) was obtained by centrifuging the blood at 900 rpm (150*g*) for 10 min at room temperature. After the final isolation step, the platelet counts were determined electronically after a 1:3,000 dilution (Model ZBI, Coulter Electronics, Hialeah, FL USA). The fatty acid compositions of the platelet membranes after dietary treatment were determined by gas-liquid chromatography analyses of methyl esters as described in a previous report from our laboratory.[6]

Chlortetracycline experiments

CTC was selected as the probe to detect the release of calcium from internal membranes in thrombin-stimulated platelets. The characteristic of the CTC-calcium complex to demonstrate a decrease in fluorescence as it shifts from a nonpolar environment to a more polar one makes it an excellent indicator of calcium release from membranes to cytosol.[9] Platelets were isolated and washed prior to being loaded with this probe according to a procedure adapted from Jy and Haynes.[9] EGTA (2 mM) and PGI_2 (0.25 μM) were added to the PRP, and the platelets were isolated from the plasma by centrifugation at 10,000 rpm for 90 sec at room temperature in a microcentrifuge (n = 4/diet). The cells were washed and suspended in a calcium- and magnesium-free Tyrode's buffer containing 138 mM NaCl, 3 mM KCl, 10 mM glucose, 2 mM $NaHCO_3$, 0.4 mM $NaH_2PO_4 \cdot H_2O$, and 2.5 mM HEPES, adjusted to a pH of 7.3.[9] Platelets (10^8 cells/mL), suspended in the modified Tyrode's buffer, were preincubated for 15 min at 37°C in a cuvette, followed by the addition of CTC (40 μM), which was incubated for an additional 15 min at 37°C. Then the cuvette, containing a Teflon-coated microstirring bar, was introduced into the instrument. Fluorescence measurements were made for 5 min in order for equilibrium to be established, followed by the addition of thrombin (0.25 to 1.0 U/mL). Measurements were monitored in a fluorimeter (Model 4800S, software version 3.2, SLM Instruments, Inc., Urbana, IL USA) at an excitation wavelength of 390 nm and an emission wavelength of 530 nm in a thermostatically controlled cell holder using excitation and emission slits of 8 nm. To minimize light scattering effects, the excitation beam was polarized horizontally[11] and a 470 nm cut-off filter was employed on the emission beam. The slope of the fluorescence decrease was calculated as the difference from the point of thrombin injection to the minimum point of the slope divided by the change in the time in seconds. Four animals from each dietary group were used in these CTC experiments.

Aequorin loading

Aequorin, the photoprotein that emits a luminescent signal when it binds calcium, was used to monitor the homeostatic fluxes of this ion in resting platelets. This protein has been shown to be uniquely sensitive to local calcium transients within the cell; therefore, monitoring the decay of the signal over time would reflect exposure of aequorin to basal fluxes of this ion.[10] For these experiments, PRP was centrifuged in the presence of 0.25 μM PGI_2 at 10,000 rpm for 90 sec at room temperature. The platelet pellets were resuspended and washed with a HEPES buffer consisting of 140 mM NaCl, 2.7 mM KCl, 3.8 mM HEPES, 0.1% glucose, and 0.1% albumin, also containing 1.9 mM EGTA and 0.25 μM PGI_2 at a pH of 7.1. The cells were centrifuged at 10,000 rpm for 90 sec. Pellets were resuspended in 90 μL of HEPES buffer with 0.25 μM PGI_2, and 10 μL of aequorin (2 mg/mL) was added as adapted from Yamaguchi et al.[12] Then 0.9 μL of DMSO was introduced every minute for 6 min (final DMSO concentration was 4%, instead of the usual 6%, and total volume of DMSO added was 6.3 μL). After the final DMSO addition, platelet suspensions were allowed to stand for 1.5 min before being diluted to 1.0 mL with the HEPES buffer. Cells were filtered through a Sepharose 2B column pre-equilibrated with incomplete Tyrode's buffer containing 136 mM NaCl, 2.7 mM KCl, 0.86 mM $NaH_2PO_4 \cdot 2H_2O$, 12 mM $NaHCO_3$, 0.25 μM PGI_2, 0.1% albumin, and 0.1% glucose at a pH of 7.1, a procedure that was modified from that described by Lages and Weiss.[13] The platelets were collected using this solution as the elution buffer.

Aequorin decay

To determine total aequorin luminescence (L) and aequorin decay, 2×10^7 platelets/mL were incubated at room temperature under the following conditions prior to lysis: exposed to 0.3 mM of Mg^{2+} and 0.7 mM of Ca^{2+} for 30 min, exposed to 0.3 mM Mg^{2+} and 1.5 mM of Ca^{2+} for 30 min, and exposed to 0.3 mM of Mg^{2+} and 0.7 mM of Ca^{2+} for 5 min just prior to all lysis steps. Aliquots of the platelet suspensions were preincubated for 2 min at 37°C in the PICA® (Platelet Ionized Calcium Aggregometer, Chrono-log Corp.). The aliquots, which were stirred at 1,200 rpm

in the instrument, were then lysed with Triton X-100 (0.05%) at 0, 9, 16, 25, and 30 min. Aequorin luminescence (L) was determined as the difference from the baseline to the peak maximum (cm) following the addition of Triton X-100. Nine animals from each dietary group were used for the aequorin experiments.

LDH measurement

To assess maintenance of platelet membrane integrity, lactate dehydrogenase (LDH) activity was determined in supernatants after loading with the cells with aequorin and varying amounts of DMSO using a standard spectrophotometric assay.[14] LDH release from the aequorin-loaded platelets was less than 5% when 4% DMSO was used. Loading aequorin with a final DMSO concentration of 6% was found to be unacceptable because it caused an LDH release of greater than 10%.

Total calcium determination

Total calcium in rat platelets was determined by an atomic absorption procedure[15] using a flame absorption spectrometer (Model 5000, Perkin-Elmer Corp., Norwalk, CT USA). Total calcium in the deionized water was analyzed with a calcium electrode (Orion Research Inc., Boston, MA USA).

Statistical analysis

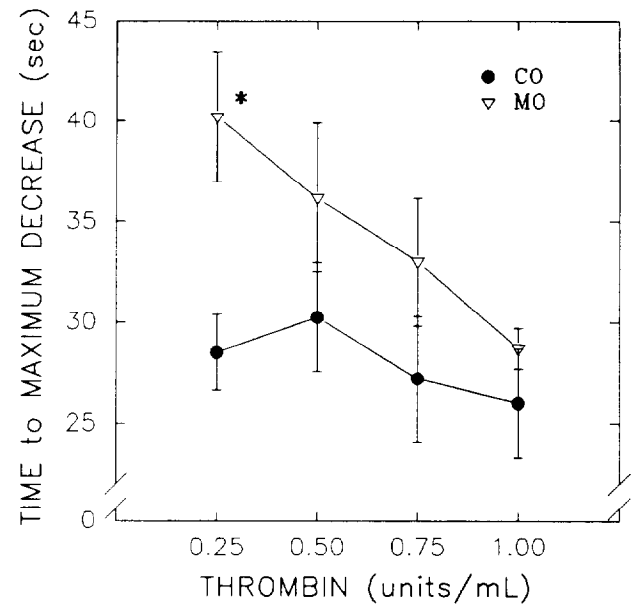
The data were analyzed by means of a Student's *t*-test. A result was considered to be significant if the *p*-value was less than 0.05.

Results

After feeding the CO and MO diets for 8 to 12 days, the fatty acid compositions of the platelet phospholipids reflected dietary intake as expected and corresponded to previous results reported by our laboratory.[6] Eicosapentaenoic acid (EPA, 20:5n-3) increased from 0.4% on the CO diet to 6.5% on the MO diet, and arachidonic acid (AA; 20:4n-6) decreased from 17.7% on the CO diet to 12.4% on the MO diet.

In order to evaluate the effect of an n-3 PUFA (MO) versus an n-6 PUFA (CO) fatty acid diet on the ionized calcium release from intracellular storage compartments, the time to maximum decrease in CTC fluorescence was monitored after the addition of thrombin to platelets from rats fed either of the diets.

Because these experiments were designed to monitor the release of calcium from internal stores, they were conducted in the presence of the lowest amount of external Ca^{2+} possible (2×10^{-5} M) in the resuspending buffer solution without adding a chelator. This concentration was determined with a calcium electrode and confirmed by atomic absorption analysis. Time constraints allowed two platelet samples per day, one from each dietary group ($n = 4$), to be loaded with CTC as described in the Materials and Methods section and then analyzed for fluorescence decreases initiated by four different concentrations of thrombin. *Figure 1A* shows a plot of times required to achieve the maximum change in CTC fluorescence in platelets isolated from the two groups of animals for the four different doses of thrombin used. A typical curve representative of the decrease in fluorescence seen with thrombin addition is included (*Figure 1B*). For platelets from rats fed CO, there



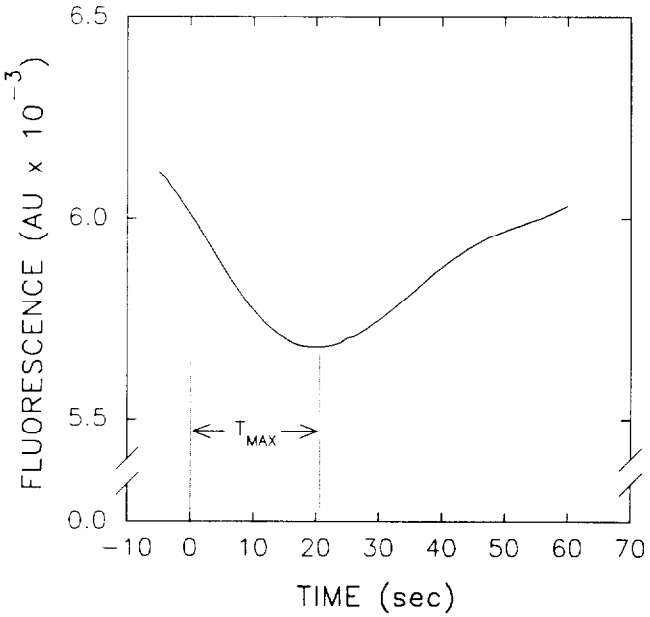


Figure 1 (A) Times for the maximum decrease in fluorescence were plotted against the concentrations of thrombin used. Values shown represent the mean ± the standard error of the mean (SEM) from platelets of rats fed either CO (●) or MO (▽) at 0.25, 0.50, 0.75, and 1.0 U/mL of thrombin, $n = 4$. *$P = 0.02$, CO vs. MO (0.25 U/mL thrombin). $r = 0.78$ for a regression line through values for the MO diet. (B) The curve is representative of the decrease in fluorescence seen with thrombin and illustrates how time for maximum decrease (T_{MAX}) was measured from the point of addition to the minimum point observed.

was no observed dose-dependent response to the four different concentrations of thrombin. The time for maximum decrease in CTC fluorescence remained relatively constant as the concentration of thrombin was increased from 0.25 to 1.00 U/mL. However, for platelets from rats fed MO, it

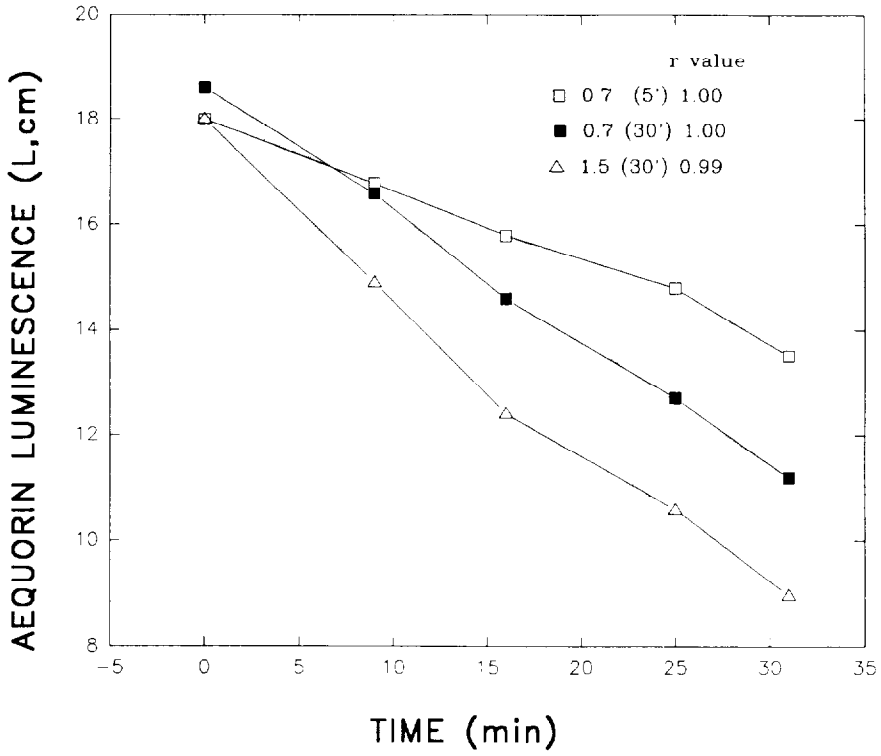


Figure 2 The decay of aequorin in platelets from rats fed a standard laboratory diet (mean plotted, $n = 9$). Platelets were exposed to either 0.7 or 1.5 mM of Ca^{2+} for 30 min, or to 0.7 mM Ca^{2+} for 5 min prior to lysis. Aliquots of the cells were incubated for 2 min at 37°C and then lysed by the addition of Triton X-100 (0.05%).

took longer for the CTC signal to reach the maximal decrease for the lowest dose of thrombin compared with the time observed for platelets from rats fed CO. The difference in response time to this dose of thrombin was significant between the dietary treatments ($P = 0.02$). As the dose of thrombin increased, times decreased for the n-3 PUFA-enriched platelets in a linear ($r = 0.78$) dose-dependent manner and became similar to those for the n-6 PUFA control platelets.

To establish appropriate conditions for observing decreases in the luminescent signal, aequorin decay in platelets from rats fed a standard laboratory diet was studied at two different concentrations of extracellular Ca^{2+} (*Figure 2*). Two aliquots of gel filtered platelets were constantly exposed to either 0.7 or 1.5 mM of Ca^{2+} for 30 min. A third aliquot was maintained in a nominal amount of Ca^{2+}, and the extracellular Ca^{2+} was brought to 0.7 mM just prior to lysis. There were no significant differences in the decay rates between the platelets that were constantly exposed to either 0.7 or 1.5 mM Ca^{2+}, and total aequorin luminescence decreased linearly over 30 min, $r = 1.00$ for 0.7 mM Ca^{2+} (5 and 30 min exposure) and $r = 0.99$ for 1.5 mM Ca^{2+}. The decay rates at 30 min were significantly different between the platelets that were constantly exposed to Ca^{2+} and those maintained without the added Ca^{2+} (*Table 1*).

For the aequorin experiments, platelets from rats fed CO and MO were loaded with this probe, and the decay rate measurements were completed within 30 min (two platelet preparations/day, one from each group). Since loading the cells with aequorin required a significant amount of handling of the platelets, all precautions were taken to ensure that both sets of cellular suspensions were treated equally. Amounts of total platelet calcium were not affected by diet and fell within the expected range based on the analyses by atomic absorption (data not shown). A linear decrease in the total aequorin luminescence was also observed over 30 min (*Table 2*) for platelets isolated from the experimental dietary groups. *Figure 3* shows the results of the aequorin decay at the two different concentrations of extracellular Ca^{2+} (0.7 and 1.5 mM) for platelets from animals fed either CO or MO. Within the same dietary group, no significant difference was observed in aequorin decay in platelets exposed to either concentrations of external Ca^{2+}. However, a significantly lower decay rate was observed at both concentrations of extracellular Ca^{2+} for platelets from animals fed fish oil ($P = 0.003$).

Discussion

In this report, we have described our results obtained with two different reporter molecules whose unique properties

Table 1 Aequorin decay rates in platelets from rats fed a standard laboratory diet

$[Ca^{2+}]$ (mM)	Exposure time (min)	Decay* (mm/min)	*P* value (5 vs. 30 min)
0.7 ($n = 8$)	5	14.5 ± 0.69	—
0.7 ($n = 14$)	30	24.1 ± 1.56	0.0004
1.5 ($n = 13$)	30	29.0 ± 1.67	0.00001

*Shown is the mean ± SEM. Platelets (2×10^7/mL) were either exposed to extracellular Ca^{2+} (0.7 and 1.5 mM) or incubated in the presence of a nominal amount of Ca^{2+} (2×10^{-5} mM; see Materials and Methods section for experimental details), and the ion was added to the extracellular media 5 min prior to lysis.

Table 2 Linearity of aequorin decay in the diets

Diet	$[Ca^{2+}]$ (mM)	r
CO	0.7	0.99
	1.5	0.99
MO	1.7	0.99
	1.5	0.98

Platelets (2×10^7 cells/mL) from rats fed either a CO or MO diet were isolated and exposed to either 0.7 or 1.5 mM of Ca^{2+} for 30 min and then lysed ($n = 9$, see Materials and Methods section for experimental details).

were used to investigate the effects of n-3 PUFA versus n-6 PUFA on systems controlling cytosolic Ca^{2+} under stimulated and resting conditions. The probe CTC has been used predominantly to provide information regarding Ca^{2+} movements in intracellular storage organelles in platelets.[16–19] However, we are the first to use this probe to study the interactions of dietary PUFA with intracellular calcium stores. We used CTC to monitor stimulated calcium release from intracellular storage compartments in platelets from rats fed either CO or MO diets. In the presence of a nomimal concentration of extracellular ionized calcium, the time required to maximally release the calcium ion from the intracellular stores remained relatively constant in the platelets from rats fed CO, while the n-3 PUFA-enriched platelets showed a dose-dependent decrease in the time required to mobilize Ca^{2+} as the amount of thrombin was increased (*Figure 1*). Furthermore, a significant difference in this decrease between the diets occurred at the lowest concentration of thrombin.

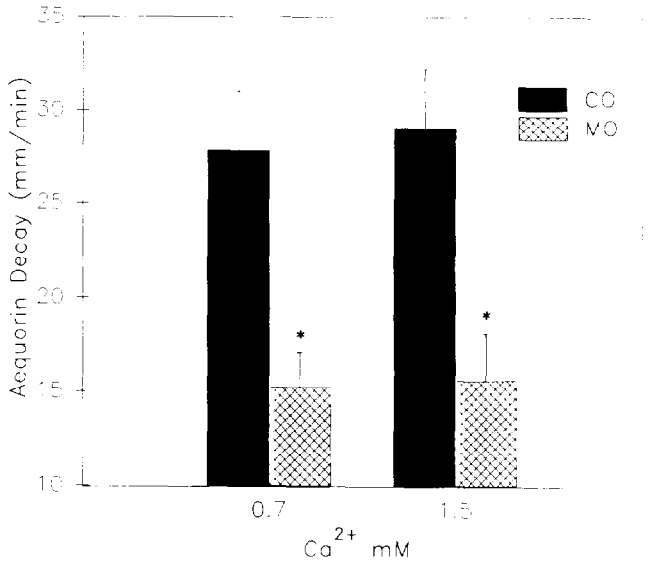


Figure 3 The decay of aequorin was determined as the difference in luminescence from the initial peak lysis, to the lysis at 30 min, divided by the time (mm/min). Shown is the mean ± SEM, $n = 9$. Experiments were conducted at two concentrations of extracellular Ca^{2+} (0.7 and 1.5 mM) with platelets from rats fed either CO or MO. *$P = 0.003$, CO vs. MO, at both calcium concentrations.

CTC has been used to show that resting levels of Ca^{2+} in the intracellular storage compartments in platelets from patients with arterial thrombosis are higher than levels in platelets from normal donors.[18,19] It has been proposed that both increased membrane-bound and cytosolic Ca^{2+} concentrations in the resting state renders platelets from patients with thrombotic disorders more sensitive to stimulation by physiologic agents, that is, more responsive to low agonist concentrations.[18] Thus conditions that favor a slower generation of Ca^{2+} signal would result in decreased responsiveness even in the presence of disease-driven platelet hyperactivity. Our results suggest that n-3 fatty acids, by promoting mechanisms that delay or possibly inhibit the release of calcium from intracellular organelles, would reduce the responsiveness of platelets and decrease thrombotic tendency. Since we did not measure stimulated thromboxane A_2 production or use cyclooxygenase inhibitors, it is not clear from the CTC experiments what contributions are made by subtle changes in eicosanoid production to the kinetics of Ca^{2+} signaling.

Aequorin decay in platelets from rats fed a standard laboratory diet was linear over 30 min whether cells were incubated in a buffer containing a relatively low Ca^{2+} concentration or whether they were exposed to extracellular Ca^{2+}. The decrease in luminescence per minute was significantly different between the platelets that were incubated with and without Ca^{2+}, as determined at 30 min. These data, however, do not agree with those reported by Malmgren et al.[20] These investigators observed no significant differences in the decay rates of aequorin in human platelets incubated with or without Ca^{2+} after 60 min. Whether the contrasting results are due to species differences or to variations in experimental conditions between laboratories is unknown. One dissimilarity is the technique employed for loading aequorin into the platelets. We used DMSO at a final concentration of 4%, as compared with the 6% used by the other group.[20] We used the lower percentage of DMSO because the higher percentage produced a significant release of LDH, indicating a substantial loss of cellular integrity. Our data indicated no difference in the decay rate when the platelets were constantly exposed to either 0.7 or 1.5 mM of Ca^{2+}. The significant difference in decay rates observed in this study between platelets maintained in the presence or absence of external Ca^{2+} is evidence that the aequorin signal is sensitive to extracellular Ca^{2+} as previously suggested by Lages and Weiss.[21] These investigators imply that the higher decay rates for aequorin in platelets exposed to Ca^{2+} indicates that the photoprotein is located near the plasma membrane or pump leak sites and can readily react with nearby Ca^{2+}. This location characteristic allows this probe to report information on calcium movements across the plasma membrane.

Basal conditions established for aequorin decay with platelets from rats maintained on standard laboratory rations were applied to platelets from the animals fed the experimental diets. The decay rates for aequorin in platelets enriched by dietary n-3 PUFA were significantly lower than those in platelets containing n-6 PUFA for both concentrations of Ca^{2+} used. One explanation for this difference is that n-3 PUFA and n-6 PUFA in platelet lipids may differ-

entially modulate the activity of receptor-operated calcium channels in the plasma membrane. Recently, Hornstra et al.[22] have suggested that dietary fatty acids may mediate platelet aggregability in rats by modulating transmembrane calcium flux. Two recent studies by Hallaq et al.[22,23] with rat cardiac myocytes have demonstrated that incorporation of n-3 PUFA into membranes decreased calcium movements within and into cells using fura-2 to detect Ca^{2+}. The initial study with neonatal rat myocytes indicated that replacement of arachidonic acid in membrane lipids by eicosapentaenoic acid protected the cells from the toxic effects of ouabain by preventing the excessive increase in cytosolic ionized calcium provoked by the cardiac glycoside.[23] In a subsequent report,[24] these same investigators demonstrated that the protective effect of the n-3 PUFA was related in part to a reduction in calcium influx through channels in the plasma membrane of the myocytes. Arachidonic acid demonstrated no such action when compared with n-3 PUFA at the low concentrations of fatty acids used in these experiments (5 μM). The diverse effects of arachidonic acid and n-3 PUFA on cytosolic calcium signals is further supported by experiments demonstrating a potentiating effect of exogenously added arachidonic acid itself and not its metabolites on the generation of this signal in rat cardiac myocytes.[25]

Our observations that n-3 PUFA can affect both intracellular release and transients across membranes in platelets are essentially in agreement with the in vitro studies with myocytes reported by Hallaq et al.[23,24] In contrast, no decreases were observed in Ca^{2+} signals in collagen-stimulated platelets from humans who had received supplements comparing intake of ethyl eicosapentaenoate or fish oil to ethyl oleate.[26] Even though the stimulated increases in platelet cytosolic Ca^{2+} were the same for the three treatments as measured by aequorin, supplementation with n-3 PUFA did result in reduced aggregation and thromboxane A_2 synthesis in platelets of the subjects. Variations among laboratories in the isolation and preparation of platelets for in vitro studies may contribute to the sometimes conflicting results reported by investigators and could be an explanation for such divergent effects. It has also been suggested that aequorin may not be the best probe to determine receptor-mediated Ca^{2+} increases.[21]

In conclusion, we present evidence that long chain n-3 PUFA may delay the release of intracellular ionized calcium following the receptor-mediated stimulation in platelets. This was especially evident at low concentrations of agonist and may be another mechanism to explain the protective role of fish oils in the progression of cardiovascular disease. In addition, n-3 PUFA lowered calcium movements across the plasma membrane even under basal conditions. Both of these effects cooperatively interacted to depress Ca^{2+} signals and decrease platelet responses to thrombotic challenges.

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