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Pharmacology

Modulation of dihydropyridine-sensitive calcium channels in heart cells by fish oil fatty acids

(cardiac arrhythmias/ouabain toxicity/$n-3$ fatty acids/eicosapentaenoic acid/docosahexaenoic acid)

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ABSTRACT **The highly unsaturated $n-3$ fatty acids from fish oils, eicosapentaenoic acid [EPA; C20:5 ($n-3$)] and docosahexaenoic acid [DHA; C22:6 ($n-3$)], prevent the toxicity of high concentrations of the cardiac glycoside ouabain to isolated neonatal rat cardiac myocytes. Arachidonic acid [C20:4 ($n-6$)] lacks such protective action. The protective effect of the $n-3$ fatty acids is associated with their ability to prevent high levels of cytosolic free calcium from occurring in response to the ouabain. This in turn results, at least in part, from a 30% reduction in calcium influx rate induced by the $n-3$ fatty acids. This protective effect is simulated by nitrendipine, a dihydropyridine inhibitor of the L-type calcium channels in cardiac myocytes. Nitrendipine (0.1 nM) alone, however, inhibits myocyte contractility, as do verapamil (10 μM) and diltiazem (1.0 μM). EPA or DHA (5 μM) blocks the inhibitory effects of nitrendipine but not those of verapamil or diltiazem. Bay K8644, a known dihydropyridine agonist of L-type calcium channels, produces a ouabain-like effect that is also prevented by EPA or DHA. Specific binding of [^{3}H]nitrendipine to intact myocytes is noncompetitively inhibited by EPA or DHA in a manner that reduces the number of high- and low-affinity binding sites (B_{max}) and increases their affinities. The fish oil fatty acids prevent calcium overload from ouabain and Bay K8644. They also prevent a calcium-depleted state in the myocytes caused by the L-type calcium channel blocker nitrendipine. The protective effects of the $n-3$ fatty acids appear to result from their modulatory effects on nitrendipine-sensitive L-type calcium channels.**

McLennan *et al.* (1, 2) have demonstrated that fish oil feedings essentially prevented ventricular fibrillation from occurring during ischemia and reflow produced by permanent or temporary experimental occlusion of a coronary artery in rats and in monkeys. We have examined the effect of the highly unsaturated, long-chain $n-3$ fatty acids of fish oil, eicosapentaenoic acid [EPA; C20:5 ($n-3$)] and docosahexaenoic acid [DHA; C22:6 ($n-3$)], on another arrhythmogenic stress—namely, ouabain toxicity, which can be studied conveniently *in vitro* in isolated neonatal rat cardiac myocytes. We have reported that EPA, when incorporated in phospholipids of the cell membranes, prevented the toxicity caused by ouabain (0.1 mM) (3). This protection was associated with less rise in cytosolic free calcium. The present study explores the means by which this protection is effected and indicates that the fish oil fatty acids have a dual modulatory action on dihydropyridine-sensitive calcium channels. They prevent excessive or deficient influx of calcium from compromising the contractility of cardiac myocytes.

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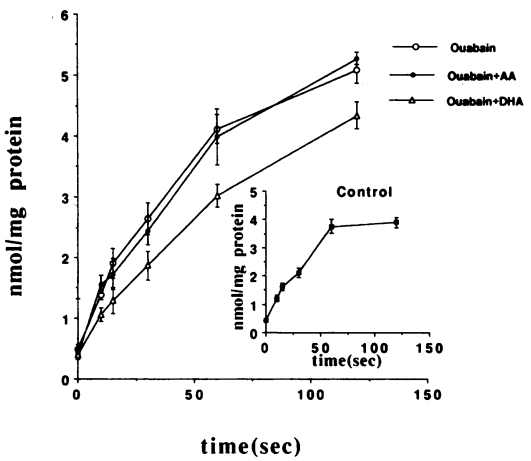

FIG. 1. Calcium uptake in control cardiac myocytes and in myocytes exposed to ouabain (0.1 mM) after incubation for 4 days with no fatty acids or with 5 μM arachidonic acid (AA) or 5 μM DHA added to the medium. Calcium uptake was measured after addition of tracer $^{45}Ca^{2+}$ to the 1.0 mM calcium in the bathing medium. Calcium uptake is shown in the presence of ouabain (0.1 mM). (*Inset*) Calcium uptake by untreated control cells. At 30 sec with ouabain alone and with ouabain plus DHA, the uptakes were 2.65 ± 0.26 and 1.87 ± 0.24 nmol/mg of protein, respectively ($P < 0.025$). The number of experiments for each point was 6–15 for the control, 6–9 for ouabain, 4–9 for AA, and 4–11 for DHA.

METHODS AND MATERIALS

The isolation and culture of cardiac myocytes of 1- to 2-day-old rats, as well as the measurements of contractility, were performed as described (3). Calcium flux into the isolated spontaneously beating cardiac myocytes was determined with tracer concentrations of $^{45}Ca^{2+}$ in the presence of 1.0 mM calcium in the bathing medium. To obtain steady-state values for calcium influx, measurements were made after 7 min of exposure of the cells to agonists or antagonists. The initial uptake of $^{45}Ca^{2+}$ by the myocytes 0, 10, 15, 30, 60, 90, and 120 sec after exposure to the $^{45}Ca^{2+}$ was measured to estimate rates of calcium influx (4). Cells adherent to tissue culture dishes were incubated in a Hepes/saline solution (140 mM NaCl/5 mM KCl/1.0 mM $MgCl_2$/1.0 mM Na_2HPO_4/5 mM Hepes/10 mM glucose, adjusted to pH 7.4 with NaOH). At the specified time following addition of the $^{45}Ca^{2+}$ to the bathing solution, the cells were rinsed rapidly four times with 3 ml of cold (4°C) nonlabeled buffered medium and then

Abbreviations: EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; AA, arachidonic acid.

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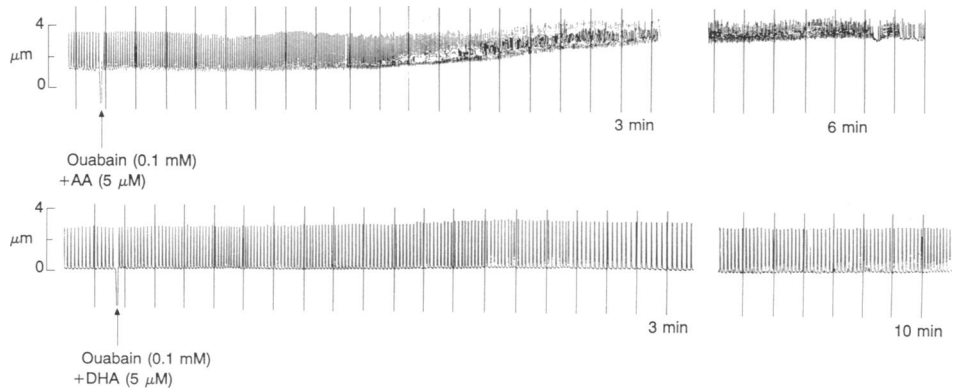

FIG. 2. DHA (5 μM), but not AA (5 μM), prevented ouabain toxicity when added to the medium bathing the cardiac myocytes together with the ouabain. The same protective effect is seen as occurs when cells are enriched with DHA (5 μM) in culture. (n = 10 experiments.) The time between the vertical bars in this and other tracings is 10 sec.

lysed; aliquots were taken for scintillation counting and determination of protein content.

Nitrendipine binding studies were made with the intact spontaneously beating cardiac myocytes by using [^{3}H]nitrendipine at a tracer concentration of 0.03 nM and were done in parallel with contractility measurements on cells from the same batch of myocytes. The myocytes were incubated with a constant concentration of the [^{3}H]nitrendipine and with nonradioactive nitrendipine added to yield total concentrations of nitrendipine from 0.03 to 10 nM in the Hepes/saline solution. The nonspecific binding was obtained with concentrations of unlabeled nitrendipine of 100 μM, and this value was subtracted from the total binding obtained at lower concentrations of nitrendipine to obtain the specific binding. The "zero time" tissue uptake of [^{3}H]nitrendipine (0.03 nM) on three determinations was 13%, 11%, and 11% of the total tissue radioactivity. The retention of labeled nitrendipine in the reaction dish after a 60-min exposure was 8% and 4.7% of total tissue radioactivity without or with 100 μM nonlabeled nitrendipine, respectively. All observations on cells were made at 37°C.

All reagents were purchased from Sigma. Bay K8644 was obtained from Calbiochem, and nitrendipine was purchased from Miles. The [^{3}H]nitrendipine (specific activity of 84 Ci/mmol; 1 Ci = 37 GBq) and the $^{45}Ca^{2+}$ were purchased from New England Nuclear.

Significance of differences between two sample means was evaluated with Student's t test. Nitrendipine binding data were analyzed by the LIGAND program (5). Data are expressed as the mean ± SEM, and significant differences were accepted at $P < 0.05$.

RESULTS

Effect of $n - 3$ Fatty Acids on Ca^{2+} Influx. Fig. 1 shows that the protective effect of the fish oil fatty acid DHA on intracellular calcium concentration ($[Ca^{2+}]_i$) results, at least in part, from a reduction in Ca^{2+} influx. Fig. 1 shows that in cells exposed to ouabain (0.1 mM) with no added fatty acid or in cells incubated for 4 days with 5 μM AA the initial rate of $^{45}Ca^{2+}$ uptake was higher by some 30% than when the cells had been enriched with 5 μM DHA. Uptake of $^{45}Ca^{2+}$ is essentially linear over the first 30 sec, and the differences in the uptake curves are significant by that time. This finding is consistent with the lower $[Ca^{2+}]_i$ levels found following ouabain exposure in the myocytes protected by the $n - 3$ fatty acids (3).

Acute Effects of $n - 3$ Fatty Acids on Ouabain Toxicity. To this point, all studies were performed with EPA or DHA enrichment of membrane phospholipids. When the free fatty acids were added directly to the buffered medium bathing the cells together with the ouabain, however, the $n - 3$ fatty acid (either EPA or DHA) exhibited the same protective effect against ouabain toxicity as when the cells had been incubated 3–5 days in the presence of the $n - 3$ fatty acid (Fig. 2). Furthermore, DHA (5 μM) may be added 1–2 min after addition of the ouabain and still block the ouabain toxicity (data not shown).

Effect of a Dihydropyridine Calcium Channel Inhibitor on Ouabain Toxicity. Since the $n - 3$ fatty acids reduced $^{45}Ca^{2+}$ influx, and this protected the myocytes from ouabain toxicity, the effect of a known calcium channel blocker on ouabain toxicity was tested. As shown in Fig. 3, nitrendipine, even at 0.1 nM, not only prevented the toxic state of contracture

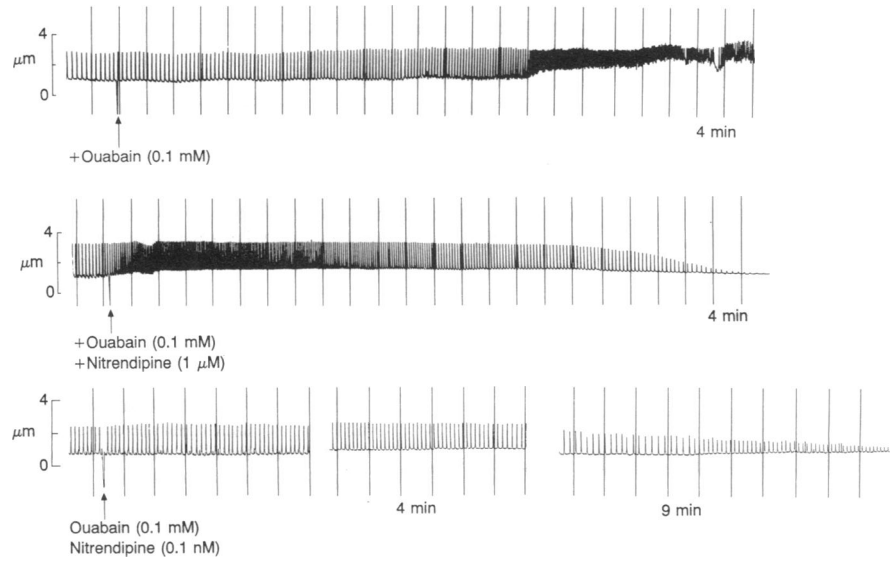

FIG. 3. Nitrendipine (1 μM) prevents ouabain toxicity similar to the $n - 3$ fatty acids. Nitrendipine suppressed contractility without contracture. (n = 7 experiments.)

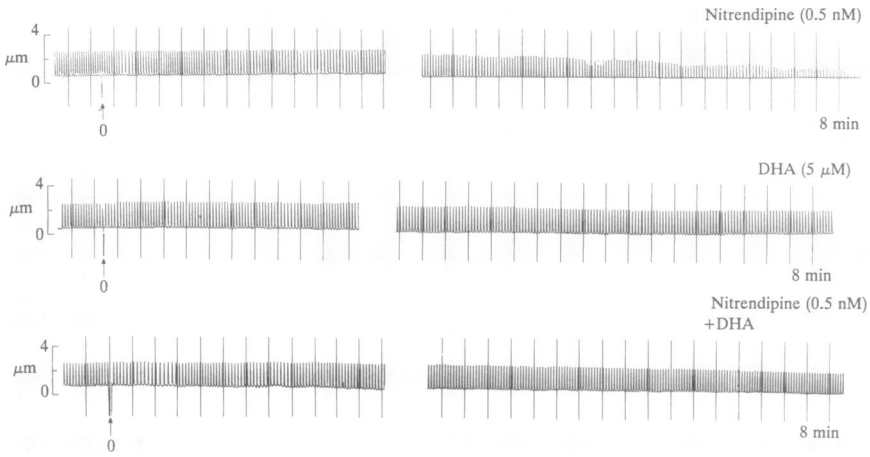

FIG. 4. DHA (5 μM), which did not affect contractility, prevents the suppression of myocyte contractility caused by nitrendipine (0.5 nM). (n = 6 experiments.)

caused by ouabain but reduced myocyte contractions in a state of diastolic relaxation. Since nitrendipine is a known inhibitor of the slow calcium channels in heart cells (6–8), this result suggests that the $n-3$ fatty acids may also be affecting the same channels.

Interactions of Nitrendipine and $n-3$ Fatty Acids on Contractility of the Cardiac Myocytes. Since both nitrendipine and $n-3$ fatty acids prevented ouabain toxicity, possible interactions between these two chemically distinct inhibitors of calcium influx were probed. Fig. 4 shows the effect of nitrendipine alone, DHA alone, and the two agents together on contractility of the myocytes. Nitrendipine (0.5 nM) alone clearly inhibited contractility. DHA (5 μM) alone had very little effect. When the myocytes were exposed to the two agents together, however, DHA blocked the inhibitory effect of nitrendipine.

Interaction of Bay K8644 and $n-3$ Fatty Acids on the Contractility of the Cardiac Myocytes. Bay K8644 at a low concentration (0.1 μM) is a known agonist of the L-type calcium channels (9, 10). It was, therefore, of interest to see whether by itself it would mimic the toxicity of ouabain and, if so, whether addition of DHA could eliminate its toxicity as it does with ouabain. Fig. 5 shows the effect of 0.1 μM Bay K8644 (racemic mixture) alone on myocyte contractility. There is a definite decrease in diastolic relaxation of the myocyte, as indicated by the elevation of the baseline of the tracing. A modest increase in the beating rate and a small but definite decrease in amplitude of contractions also occurred. The response to Bay K8644 was distinctly less pronounced than to ouabain.

When 5.0 μM DHA was added with the Bay K8644, the effects of Bay K8644 on contractility were nullified (Fig. 5, lower tracing). This is consistent with the ability of EPA and DHA to reduce the increase in $^{45}Ca^{2+}$ influx observed with Bay K8644 alone (Table 1).

Lack of Effect of $n-3$ Fatty Acids on the Actions of Verapamil or Diltiazem. Since both verapamil and diltiazem are known to block calcium influx through the L-type calcium channels, their actions were also tested in the presence and absence of DHA (5 μM) added to the medium bathing the myocytes. When verapamil (10 μM) or diltiazem (1.0 μM) was added to the medium bathing the myocytes, each stopped the spontaneous contractions, as nitrendipine had done. When DHA (5 μM) was added with the verapamil (n = 3) or diltiazem (n = 4), however, it failed to prevent their inhibitory action on contractions of the myocytes. These results are consistent with the fact that verapamil and diltiazem are known to affect the L-type calcium channels at sites separate from the dihydropyridine binding site (11). Data for the verapamil and diltiazem effects are not shown.

Effects of These Agents on Calcium Influx. Table 1 shows the results of the following conditions in which $^{45}Ca^{2+}$ influx was measured: control without additives, nitrendipine [a known antagonist of the L-type calcium channels (6–8)] alone, Bay K8644 [a known agonist of the L-type calcium channels (9, 10)] alone, nitrendipine plus ouabain, and nitrendipine plus Bay K8644. It can be seen that each affects calcium influx according to expectations and that EPA and DHA, but not AA, modulate the effect of each agent. These results are consistent with the interactions shown for these agents on myocyte contractility.

Effects of the $n-3$ Fatty Acids on Binding of [^{3}H]Nitrendipine to Cardiac Myocytes. Fig. 6 shows that the specific binding of nitrendipine to the myocytes increased over a concentration range of nitrendipine of 0.03–10 nM but leveled off at the higher concentrations. Fig. 6 indicates that EPA and DHA incorporated in cell membranes noncompetitively inhibited the specific binding of nitrendipine, since EPA and DHA reduced the maximal binding of [^{3}H]nitrendipine compared with control cells or cells enriched with AA.

To further analyze the nitrendipine binding results shown in Fig. 6, Scatchard analysis (Fig. 7 and Table 2) shows that both the high- and the low-affinity binding sites (B_{max}) were reduced in number in the presence of EPA or DHA, as were the K_d values of those binding sites. Indeed, the high-affinity sites were so diminished that with DHA they were undetectable.

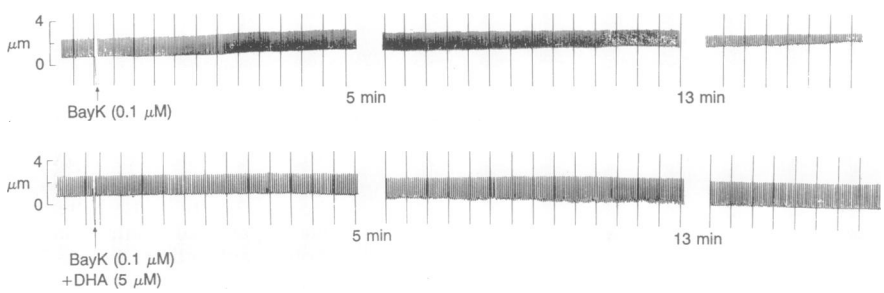

FIG. 5. Bay K8644 (BayK; 0.1 μM), an L-type calcium channel agonist, causes a failure of diastolic relaxation, decreased amplitude of contractions, and an increased beating rate of cardiac myocytes. DHA (5 μM) prevented these effects of Bay K8644. (n = 4 experiments.)

Table 1. Effects of the dihydropyridines nitrendipine (10 μM) and Bay K8644 (0.1 μM) on $^{45}Ca^{2+}$ flux into cardiac myocytes enriched with AA, EPA, or DHA or treated with ouabain (0.1 mM)

	Control		AA		EPA		DHA	
Addition	Ca^{2+} influx	% change	Ca^{2+} influx	% change	Ca^{2+} influx	% change	Ca^{2+} influx	% change
None	4.2 ± 0.2	—	4.3 ± 0.3	—	4.5 ± 0.3	—	4.4 ± 0.6	—
Nitrendipine	2.9 ± 0.2	−30	2.7 ± 0.4	−37	3.9 ± 0.5	−13	3.7 ± 0.1	−17
Bay K8644	7.6 ± 0.6	+80	7.2 ± 0.8	+68	5.3 ± 0.6	+17	5.2 ± 0.3	+18
Ouabain + nitrendipine	3.0 ± 0.1	−29	3.0 ± 0.4	−30	3.6 ± 0.5	−18	3.4 ± 0.3	−21
Bay K8644 + nitrendipine	3.1 ± 0.3	−26	3.6 ± 0.2	−17	4.3 ± 0.5	−6	4.8 ± 0.2	+9

Uptake of $^{45}Ca^{2+}$ by the myocytes after a 1.0-min exposure was used to estimate Ca^{2+} influx rates (given in nmol per mg of protein per min). The percent changes are all compared to control influx values. Each influx value is the mean ± SEM of 5–14 experiments. AA, EPA, and DHA were used at a concentration of 5 μM.

DISCUSSION

We have reported that the $n-3$ long-chain polyunsaturated fatty acid EPA of fish oil protected isolated neonatal rat cardiac myocytes from the toxic effects of ouabain (0.1 mM) and that this protection was associated with the prevention of increases in cytosolic free calcium to toxic levels (3). Here we report that measurements of the steady-state influx of calcium following exposure to the high concentration of ouabain is reduced in the presence of EPA or DHA. The effect of these fatty acids to reduce calcium influx into the myocytes must contribute to their ability to prevent ouabain toxicity. Whether they also affect the rate of calcium efflux has not been investigated. Nitrendipine, which also prevented ouabain toxicity, reduced $^{45}Ca^{2+}$ influx to a comparable degree in the presence of ouabain.

The finding that the $n-3$ fatty acids are as effective in producing the responses reported here when added acutely to the bathing medium as when the cells are initially incubated for 3–5 days in the presence of the fatty acids indicates that the effects reported are not due to physical changes in the cell membranes secondary to the incorporation of these $n-3$ fatty acids in membrane phospholipids, but rather appear to be an effect of the fatty acids on the calcium channel, either directly or—more likely—by an oxidized metabolite of the fatty acid.

The neonatal cardiac myocytes incorporated very little EPA into their membrane phospholipids during 3–5 days of incubation with 5 μM EPA in the bathing medium (3). Nevertheless, the small amounts of EPA incorporated in membrane phospholipids apparently sufficed to prevent ouabain toxicity, possibly by making available ample concentrations of the fatty acid locally at its, or its metabolite's, site of action.

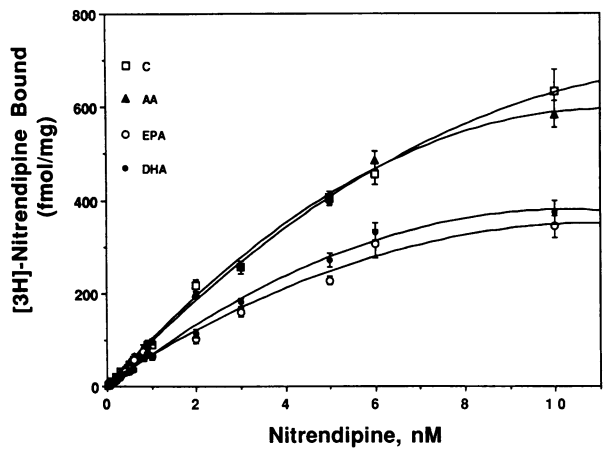

FIG. 6. Competitive displacement of [^{3}H]nitrendipine binding to the isolated cardiac myocytes by unlabeled nitrendipine with and without enrichment with AA, EPA, or DHA. The maximal binding was reduced in the presence of EPA or DHA but not AA. (n = 5–10 experiments for each point.)

DHA, which in contrast to EPA normally accumulates in phospholipids of mammalian heart cells, proved more potent than EPA in the effects we report. This indicates that its action is not secondary to its retroconversion to EPA.

The finding that DHA blocks the inhibition of myocyte contractions caused by nitrendipine but not that produced by diltiazem or verapamil suggested that both DHA and nitrendipine are affecting the same site of action. Nitrendipine, an established L-type calcium channel antagonist, inhibited $^{45}Ca^{2+}$ influx. Bay K8644, an established agonist of the L-type channels, increased $^{45}Ca^{2+}$ influx. When EPA or DHA was added with the nitrendipine, it diminished the inhibitory effect of nitrendipine on calcium influx, and when added with Bay K8644, it prevented the increase in calcium influx expected from Bay K8644 (Table 1). These effects of the fish oil fatty acids on calcium influx would account for their protective effect on myocyte contractility in the presence of either nitrendipine (Fig. 4) or Bay K8644 (Fig. 5). Finally, both the calcium influx and contractility effects result from the action of EPA and DHA to decrease [^{3}H]nitrendipine binding to its specific receptors in a noncompetitive manner. This indicates that the $n-3$ fatty acids, or their metabolites, do not bind at exactly the same sites as do the dihydropyridines but at a functionally associated site that, when occupied, can modulate the binding of the latter.

Marsh *et al.* (12) reported that the calcium channel sites affected by nitrendipine, which result in suppression of contractility in cultured chicken embryo cardiac myocytes, were those associated with the low-affinity nitrendipine binding sites rather than the high-affinity binding sites. In our results, the inhibition of the nitrendipine effect on myocyte contractility by the fish oil fatty acids was associated with a reduction in nitrendipine binding to the heart cells, which reduced apparent numbers of both the high- and low-affinity binding sites for nitrendipine (B_{max}), while increasing their affinities.

The fish oil fatty acids appear to exercise a dual effect on calcium transport; they can prevent excessive calcium influx—e.g., exposure to high ouabain concentrations or to Bay K8644—and they can enhance insufficient calcium influx—e.g., exposure to nitrendipine—when either extreme threatens to compromise the functional integrity of the cardiac myocyte. It is this apparent dual capability of EPA and DHA that requires a kinetic and molecular explanation. AA, EPA, and DHA can each be metabolized to several different compounds with distinct and often competitive and opposing actions. It is possible, therefore, that the dual action resides in the interactions of the metabolites of these $n-3$ and $n-6$ fatty acids, such that the $n-3$ metabolites compete with the formation and cellular effects of the $n-6$ metabolites and

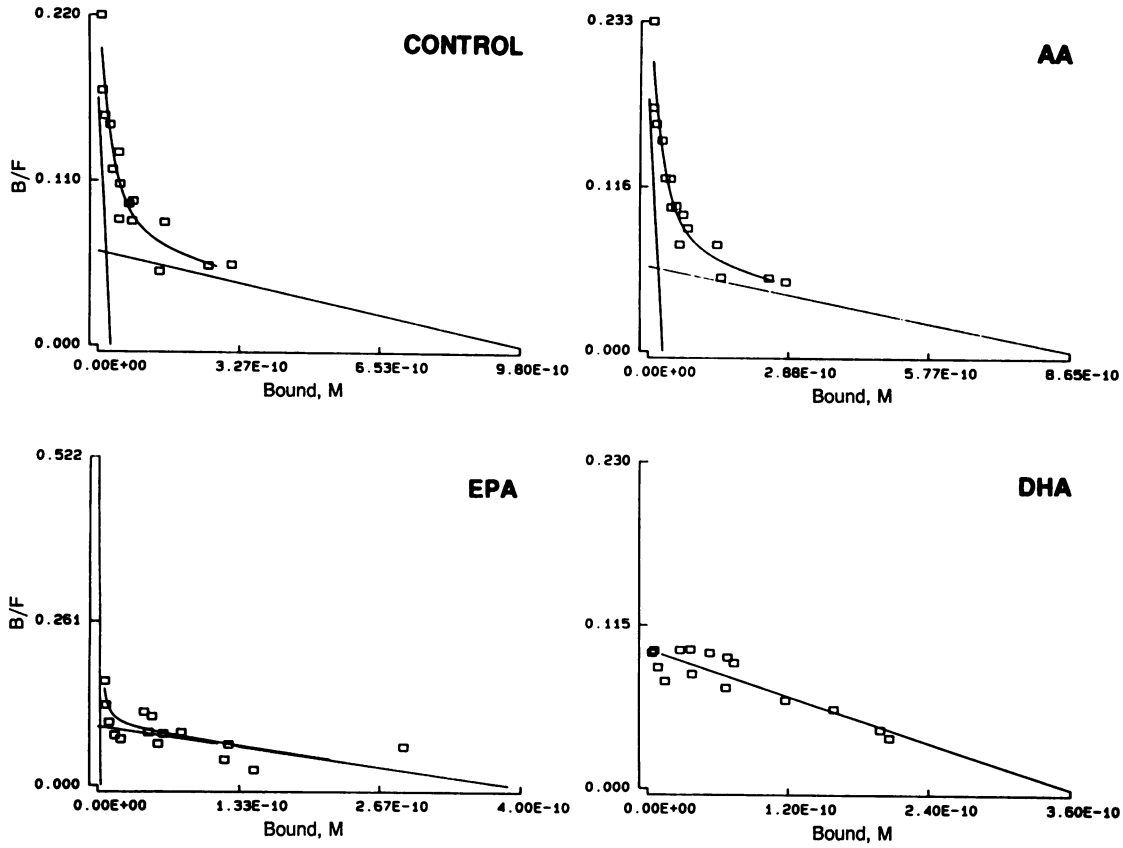

FIG. 7. Scatchard plots of the nitrendipine binding data of Fig. 6. The two-site fit is preferred for the control, AA, and EPA curves.

perhaps also convey some specific instructions of their own to the behavior of the calcium channels.

Since both ouabain and Bay K8644 increase the flux of calcium into the myocytes, a comparison was made of the effects on myocyte contractility of these two agents. Although the Bay K8644 produced changes similar to those of ouabain, full contractures and arrhythmias were not observed. Since the increase in calcium influx with Bay K8644 (0.1 μM) alone (Table 1) was greater than that with ouabain (0.1 mM) alone (Fig. 1), a more pronounced effect on contracture and arrhythmias might have been expected from Bay K8644. But since Bay K8644 should only increase entry of calcium through L-type calcium channels, whereas ouabain in addition increased sodium concentration and decreased that of potassium and cellular ATP (13), it is perhaps not surprising that the effects on contractility of these two agents differ.

The finding that these long-chain polyunsaturated $n-3$ fatty acids of fish oils can prevent the arrhythmias, fibrillation, and contracture induced by toxic concentrations of ouabain may be relevant to the reports of the ability of dietary fish oil to prevent the ventricular fibrillation associated with ischemic insults to the heart (1, 14), which cause sudden death and account for ≈60% of the annual one-half million deaths from heart attacks in the United States (15).

Table 2. B_{max} and K_d values from the Scatchard analysis of Fig. 7

	High-affinity sites		Low-affinity sites	
Addition	K_d, nM	B_{max}, fmol/mg of protein	K_d, nM	B_{max}, fmol/mg of protein
None	0.17 ± 0.06	30 ± 5	16 ± 3	980 ± 240
AA	0.18 ± 0.08	31 ± 4	14 ± 4	860 ± 150
EPA	0.005 ± 0.0007	2.9 ± 2	4.2 ± 2	390 ± 35
DHA	ND	ND	3.6 ± 0.1	350 ± 36

ND, not detectable because the DHA suppressed the high-affinity binding sites.

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