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Protective effect of eicosapentaenoic acid on ouabain toxicity in neonatal rat cardiac myocytes

(cardiac glycoside toxicity/ω-3 fatty acid effect/cytosolic calcium overload/cardiac antiarrhythmic/Na,K-ATPase inhibition)

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ABSTRACT **Isolated neonatal cardiac myocytes have been utilized as a model for the study of cardiac arrhythmogenic factors. The myocytes respond to the toxic effects of a potent cardiac glycoside, ouabain at 0.1 mM, by an increase in their spontaneous beating rate and a reduction in amplitude of contractions resulting within minutes in a lethal state of contracture. Incubating the isolated myocytes for 3–5 days in culture medium enriched with 5 μM arachidonic acid [20:4 (n-6)] had no effect on the development of lethal contracture after subsequent exposure to 0.1 mM ouabain. By contrast, incubating the myocytes for 3–5 days with 5 μM eicosapentaenoic acid [20:5 (n-3)] completely prevented the toxic effects of ouabain at 0.1 mM. There were no measurable differences in the degree to which ouabain inhibited Na,K-ATPase activity by comparing the control with the arachidonic acid- or the eicosapentaenoic acid-enriched myocytes. No differences in bumetanide-inhibitable ^{86}Rb flux were observed between the three preparations. However, measurements with fura-2 of cytosolic free calcium levels indicated that control and arachidonic acid-enriched myocytes developed toxic cytosolic calcium concentrations of 845 ± 29 and 757 ± 64 nM, respectively, on exposure to 0.1 mM ouabain, whereas in eicosapentaenoic acid-enriched myocytes, physiologic calcium levels (214 ± 29 nM) were preserved. Incubating the myocytes with eicosapentaenoic acid (5 μM) for 3–5 days resulted in a small reduction of arachidonic acid and a small but significant increase of eicosapentaenoic acid in membrane phospholipids of the myocytes.**

Epidemiologic evidence from the Greenland Eskimos (1, 2) and the Japanese (3) has suggested that eating fish and other marine animals can prevent coronary heart disease. Ingestion of fish oil affects several humoral and cellular factors that are involved in atherogenesis in a manner that may be expected to prevent atherosclerosis (for review, see ref. 4). The evidence suggests that fish oils may exert this beneficial effect by aborting the cellular pathology of atherosclerosis in the arterial wall rather than by favorably affecting the plasma lipid levels.

The animal studies by McLennan and Charnock (5, 6) have indicated an additional important cardiovascular effect of fish oils. They have demonstrated that diets high in saturated fat content increased ventricular arrhythmias induced by temporary or permanent occlusion of the coronary arteries in aged rats, whereas dietary vegetable oils diminished ventricular arrhythmias, and fish oil feeding essentially abolished fatal arrhythmias during ischemia and reflow. This antiarrhythmic effect of fish oil fatty acids was associated with increased amounts of eicosapentaenoic acid [EPA; 20:5 (n-3)] and especially of docosahexaenoic acid [22:6 (n-3)] in the sarcolemmal membranes of the heart cells. Furthermore, a prospective randomized clinical trial (7) showed that, among men who had recently suffered a myocardial infarction, those that were advised to eat fish two or three times a week had a 29% reduction in fatal myocardial infarctions over a subsequent 2-year period compared to those patients not given such advice. There was, however, no significant difference in cardiac events; those eating fish just did not die as frequently of their myocardial infarctions. This result suggests that eating fish stabilized the cardiac rhythm, preventing fatal ventricular fibrillation from occurring with the ischemic episode.

This stabilizing effect of fish oils on the heart rhythm could be the result of changes in neurogenic or circulating arrhythmogenic factors and their cardiac receptors induced by ischemia or of a direct stabilizing effect of enrichment of cardiac cells with fish oil fatty acids that is independent of extraneous factors. To distinguish between these possibilities, we studied isolated cardiac myocytes from neonatal rats *in vitro*, free from circulating agonists. These cells beat spontaneously with a regular rhythm and amplitude of contraction. They maintain their function for several days in culture medium, which allows time for incorporation of long chain polyunsaturated fatty acids into the phospholipids of their cell membranes. The effects of such membrane modifications on the function and survival of these cells can be quantitatively assessed.

We have examined another aspect of stabilization of cardiac myocyte function by (n-3) fatty acids in this preparation, namely, a preventive effect on toxicity from cardiac glycosides—another arrhythmogenic stress. These neonatal rat cardiac myocytes are sensitive to the effects of the cardiac glycoside ouabain, and at 0.1 mM ouabain the cells go into tetany and die. We report here that incorporating EPA into the cell membranes of the isolated cardiac myocytes protects them from the fatal effects of 0.1 mM ouabain, at least in part, by preventing toxic levels of cytosolic calcium from developing.

MATERIALS AND METHODS

Tissue Culture. Myocardial cells were isolated from the hearts of 1-day-old rats by serial trypsin treatment, as described by Yagev *et al.* (8). Myocytes were enriched relative to fibroblasts by preplating. For the measurement of cytosolic free calcium ($[Ca^{2+}]_i$), cells were plated on rectangular glass cover slips. Cells plated on circular cover slips were used for contractility determinations. For measurements of

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Abbreviations: Δ_4Ach, arachidonic acid; EPA, eicosapentaenoic acid; $[Ca^{2+}]_i$, cytosolic free calcium.

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ion fluxes, cells were maintained in 35-mm Petri dishes. At the time of the experiments, the cells were in a confluent monolayer and exhibited synchronous regular spontaneous contractions. Cells were enriched with EPA or arachidonic acid (Δ_4Ach) by adding the free fatty acid at 5 μM to the incubating medium for 3–5 days at 37°C. The incubating medium was Ham's F10 with 20% (vol/vol) fetal horse serum.

Analysis of Lipids. After 3–5 days of incubation, cells from the monolayer were washed three times with isotonic phosphate-buffered saline, scraped from the cover glass, and homogenized in 1 ml of ice-cold buffered saline (4°C). Lipids were extracted according to Folch *et al.* (9) and separated by the method of Kates (10). The fatty acid methyl esters were separated and quantified by gas chromatography (Hewlett-Packard) with a fused silica capillary Supelcowax 10 column (Supelco). Fatty acids were identified by comparing their retention times on the column with that of standards (13:0 and 25:0 fatty acids) and quantified by using known amounts of 23:0 fatty acids as an internal standard that were added at the methylation step.

Measurements of Contractility. Changes in the contractile state of individual cells in the monolayer were determined as the amplitude of the systolic motion of the cell. This was determined, as was the beating rate, using a phase-contrast microscope and video-motion detector as described (11). A glass cover slip with attached cultured myocytes was continuously superfused during contractility measurements with a Hepes-buffered solution containing 5 mM Hepes, 1 mM $CaCl_2$, 5 mM KCl, 140 mM NaCl, 0.05 mM $MgCl_2$, and 1% fetal calf serum. The flow rate was 1 ml/min. After a 10-min equilibration period ouabain was added to 0.1 mM to the perfusion fluid. Changes in amplitude and velocity of cell motion can be used to quantify the effects of a variety of agents on cell contractility, as demonstrated (11, 12). The contractility measurements were made on one cell per cover slip. Several cover slips were used for control Δ_4Ach- or EPA-enriched cells from each plating.

Measurement of $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ was measured with the fluorescent dye fura-2 (13). Cover slips of monolayers of attached myocytes were used after a 30-min loading with 5 μM fura-2 acetomethoxy ester. Measurements were made at 37°C in cuvettes with 3 ml of Hepes-buffered solution containing 140 mM NaCl, 5 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM glucose, 1 mM Na_2HPO_4, and 10 mM Hepes (pH 7.4). The fluorescence was continuously recorded using a Photon Technology International Delta Scan 1 spectrofluorometer (South Brunswick, NJ). The ratio of fluorescence during illumination at 340 and 380 nm recorded simultaneously was obtained and $[Ca^{2+}]_i$ was calculated as described (13). Calibration of the calcium signal was obtained by permeabilizing the cells with 10 μM digitonin to saturate the free fura-2 and subsequently adding an excess of EGTA plus manganese to determine background fluorescence.

^{86}Rb Influx Measurements. Activity of the sodium pump was estimated in cultured cardiac cells of neonatal rats as the difference in ^{86}Rb uptake seen with and without 0.1 or 1.0 mM ouabain in the bathing medium, according to the method of Panet *et al.* (14). The uptake measurements were made in Hepes-buffered solution containing 140 mM NaCl, 5 mM RbCl, 10 mM Hepes, 1 mM $CaCl_2$, 5 mM $MgCl_2$, and 10 mM glucose (pH 7.0). The influx measurements were started by adding 1.0 ml of the Hepes-buffered solution containing 2 μCi of ^{86}Rb (specific activity, 2.36 mCi/mg; 1 Ci = 37 GBq) to the monolayer of myocytes and continuing the incubation of the myocytes for 10 min at 37°C. The 10-min period was chosen because previous observations with Rb uptake in these cells had indicated that uptake was linear for at least 15 min. The uptake was terminated by washing the monolayer with ice-cold saline (4°C) and samples of cells were assayed for radioactivity and for protein content. Bumetanide-sensitive Rb^+ influx was obtained in the presence of 10 μM bumetanide to inhibit the Na^+–K^+–$2Cl^-$ channels and then by subtracting both the ouabain- and the bumetanide-resistant influx from the ouabain-resistant influx (15). Rb influx is expressed as nmol per mg of protein per min.

Na,K-ATPase Activity. The activity of Na,K-ATPase was assayed by the enzyme coupled NADH oxidation method (16) on a microsomal fraction prepared from cardiac myocytes after 3–5 days of incubation in medium without added fatty acids or medium enriched with 5 μM EPA or 5 μM Δ_4Ach. Oxidation of NADH was continuously monitored spectrophotometrically at 340 nm with and without 0.1 mM or 1.0 mM ouabain present, as a measure of the ouabain-inhibitable ATP hydrolysis by the Na,K-ATPase.

Statistical Analyses. All data are expressed as mean ± SEM. Statistical analysis of comparisons between different treatment groups was done using Student's t test; P values <0.05 were considered significant.

Materials. Δ_4Ach, EPA, boron trifluoride, methanol, lactic dehydrogenase, pyruvate kinase, standard fatty acids, and the sodium salt of NADH, grade III, were purchased from Sigma. $^{45}CaCl_2$ and ^{86}RbCl were obtained from New England Nuclear.

RESULTS

Effects of Enrichment of Membrane Phospholipids with Polyunsaturated Fatty Acids on Beating Rate and Contractility. Many studies have shown that at concentrations in the range of 0.1 mM, ouabain is a cardiac toxin that will induce arrhythmias and contracture with the heart stopping in systole. Fig. 1 shows that this toxic effect is mimicked in the isolated neonatal cardiac myocyte preparation. The top tracing is from a cell incubated 3–5 days after isolation without fatty acids added to the bathing medium. It shows that, within 6–9 min after addition of 0.1 mM ouabain to the medium, an increase in the spontaneous beating rate and a reduction in amplitude of contraction occurred. Impaired relaxation of the myocyte during diastole is seen as a rise in the diastolic baseline of the tracing. Myocytes incubated in medium-enriched with 5 μM Δ_4Ach for 3–5 days showed a similar sequence of toxic effects in response to ouabain, also developing contracture, as seen in the middle tracing. In contrast, cells that had been enriched with 5 μM EPA added to the bathing medium experienced a salutary cardiotonic effect from this concentration of ouabain, as seen by the increased amplitude of each contraction. The sagging of the diastolic baseline suggests improved relaxation of the myocyte during diastole.

The effects of the fatty acid enrichment on the amplitude of contraction and on the beating rate of the isolated neonatal rat cardiac myocytes are summarized in Table 1. Thus, EPA, an (n-3) polyunsaturated fatty acid from fish oil, when present for 3–5 days in the culture medium of the isolated cardiac myocytes, clearly protects the myocytes from toxicity during exposure to high concentrations of ouabain (0.1 mM).

Phospholipid Fatty Acid Composition in Cardiac Myocytes Enriched with Δ_4Ach or EPA. The effects on membrane phospholipid fatty acid composition of incubation in medium enriched with Δ_4Ach or EPA or control medium with no additives were examined. Isolated cardiac myocytes from 1-day-old rats were incubated for 3–5 days with no additives or with 5 μM Δ_4Ach or 5 μM EPA in the culture medium. The fatty acids of myocyte phospholipids were determined. The content of each fatty acid is expressed as μg of fatty acid per mg of protein in the sample. Results are shown in Table 2.

Δ_4Ach [20:4 (n-6)] and its elongation product 22:4 (n-6) were not increased in the fatty acids of membrane phospholipids by enrichment of the medium with Δ_4Ach, but both were significantly reduced with EPA enrichment compared with the

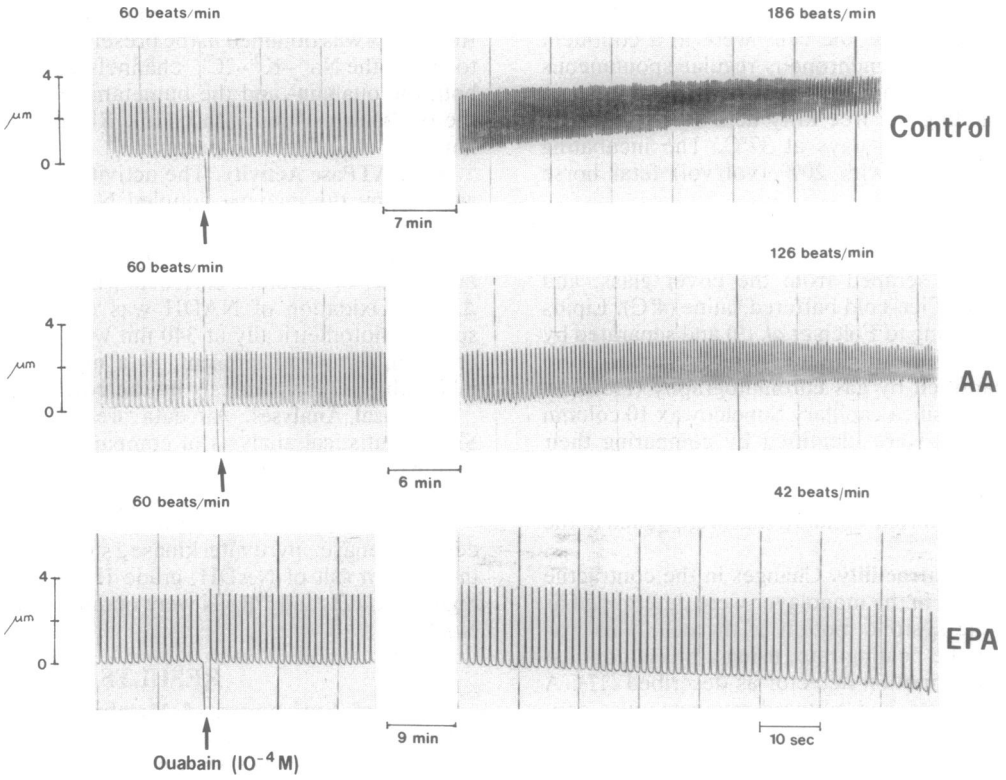

FIG. 1. Effect of 0.1 mM ouabain on the contractile state of single representative cardiac myocytes incubated 3–5 days without fatty acids added to the incubating medium (top tracing) or with 5 μM Δ_4Ach (AA; middle tracing) or with EPA (bottom tracing) present in the incubating medium. The toxic effects of ouabain are seen in the control and Δ_4Ach-enriched myocytes as an increased rate and a decreased amplitude of contractions as the myocytes proceed into lethal contracture. By contrast, the toxic effects were not exhibited by the EPA-enriched myocytes, as seen in the bottom tracing.

Δ_4Ach-enriched cells. EPA [20:5 (n-3)] was present only in small amounts in the phospholipids of neonatal cardiac myocytes and showed a small but highly significant increase with EPA enrichment, as did its elongation product 22:5 (n-3), compared with Δ_4Ach-enriched cells. Interestingly, there was no detectable increase in the desaturation products of either the (n-6) Δ_4Ach or the (n-3) EPA in the enriched myocytes. Since the myocytes were carefully washed after removal from the 3- to 5-day incubation medium prior to exposure to ouabain, we conclude that the resistance to the toxic effects of high concentrations of ouabain is closely correlated with, and probably results from, the modest changes found in the fatty acid composition of the cardiac myocytes.

Cytosolic Free $[Ca^{2+}]_i$ Changes Induced by Ouabain in Δ_4Ach- or EPA-Enriched Cells. $[Ca^{2+}]_i$ is critically important in causing concentration and relaxation of cardiac muscle cells. Thus, we examined the $[Ca^{2+}]_i$ within the isolated cardiac myocytes before and 10 min after addition of ouabain. The results are summarized in Table 3. In the absence of ouabain the time-averaged $[Ca^{2+}]_i$ levels were the same for the control cells, those enriched with Δ_4Ach, and those enriched with EPA. With exposure to 1 μM ouabain, there was a substantial increase in $[Ca^{2+}]_i$ in all preparations, but the increase was less in the myocytes enriched with EPA. At the toxic level of 0.1 mM ouabain, there was a large further increase in $[Ca^{2+}]_i$ in the control and Δ_4Ach-enriched myocytes, but no further increase in the EPA-enriched cells. This physiologic level of time-averaged $[Ca^{2+}]_i$ accounts for the preserved contractility and lack of toxicity in the EPA-enriched myocytes in the presence of 0.1 mM ouabain.

Inhibition of Na,K-ATPase. To determine the reason for the lower calcium levels and continued normal (actually enhanced) contractility of the EPA-enriched myocytes, we considered the possibility that the EPA had somehow prevented ouabain from inhibiting the Na,K-ATPase in the myocyte membrane. This hypothesis was tested by assaying Na,K-ATPase by two methods. First, the rate of influx of ^{86}Rb into the myocytes was determined as a measure of potassium influx (Table 4). The rate of the total Rb uptake expressed as μmol per mg of myocyte protein per min was the same in all preparations. The ouabain-inhibitable component of the Rb influx was also the same, as was that portion inhibitable by bumetanide. Bumetanide is an inhibitor of the facilitated cotransport pathway for Na^+, K^+, and $2Cl^-$, and

Table 1. Changes in amplitude of contraction and beating rate of neonatal rat cardiac myocytes produced by 0.1 mM ouabain

Medium addition	Amplitude, μm		Beats, no./min	
	Control	Ouabain	Control	Ouabain
None	2.76 ± 0.1 (6)	1.50 ± 0.19 (6)	72 ± 3.6 (6)	165 ± 16 (6)
Δ_4Ach	2.81 ± 0.24 (4)	1.35 ± 0.12 (4)	71 ± 2.5 (4)	161 ± 16 (4)
EPA	2.88 ± 0.13 (6)	3.84 ± 0.14 (6)	80 ± 4.0 (6)	55 ± 4 (6)

Measurements of beating rate and of amplitude of contractions were made just prior to and 6–9 min after exposure to 0.1 mM ouabain. Experimental conditions were as described in Fig. 1. Values are mean ± SEM. Numbers in parentheses are *n*.

Table 2. Fatty acid composition of phospholipids from neonatal rat cardiac myocytes, control cells, or cells enriched with Δ_4Ach or EPA

Fatty acid	Fatty acid, μg/mg of protein		
	Control	Δ_4Ach	EPA
16:0	14.0 ± 1.2	12.3 ± 0.6	11.8 ± 0.5
18:0	24.2 ± 1.5	21.8 ± 1.3	20.8 ± 0.9
18:1 (n-9)	10.6 ± 0.5	8.9 ± 0.5	8.7 ± 0.1
18:2 (n-6)	12.0 ± 0.8	10.3 ± 0.9	9.9 ± 0.3
18:3 (n-6, n-3)	0	0	0
20:4 (n-6)	21.7 ± 1.6	21.5 ± 0.8	17.5 ± 0.6*
20:5 (n-3)	0.26 ± 0.02	0.16 ± 0.03	0.5 ± 0.02†
22:4 (n-6)	4.3 ± 0.3	4.12 ± 0.2	2.9 ± 0.1*
22:5 (n-3)	2.0 ± 0.1	1.7 ± 0.1	2.7 ± 0.2†
22:6 (n-3)	5.0 ± 0.2	4.4 ± 0.3	4.2 ± 0.1

Values are means ± SEM, $n = 7$. For statistical purposes comparisons were made only between Δ_4Ach-enriched and EPA-enriched cells by partial analysis of variance. Δ_4Ach (5 μM) was added to the incubation medium for 3–5 days and EPA was added at a similar concentration and for a similar duration.

*$P < 0.002$ for reduction of 20:4 and 22:4 (n-6) in EPA-enriched myocytes.

†$P < 0.001$ for increase in 20:5 and 22:5 (n-3) in EPA-enriched myocytes.

our findings indicate that this pathway was also unaffected by enrichment with Δ_4Ach or EPA.

Second, we used the NADH-coupled enzyme assay to determine the rate of ATP hydrolysis by the Na,K-ATPase, as summarized in Fig. 2. The total ATPase activity was found to be the same in all three preparations. After exposure to 0.1 mM ouabain, a reduction of some 40% in ATPase activity was found, but without significant differences among the three preparations: control cells, cells enriched with Δ_4Ach, or cells enriched with EPA. To be certain that we had achieved full inhibition of Na,K-ATPase in all three preparations, further ATPase assays were performed in the presence of 1 mM ouabain. Though this very high concentration of ouabain did produce a modest further inhibition of ATPase activity, there were still no significant differences among the three preparations.

From these findings, we conclude that differences in $[Ca^{2+}]_i$ levels cannot be explained simply by a lesser degree of inhibition of the Na,K-ATPase in the EPA-enriched myocytes at the concentrations of ouabain used.

DISCUSSION

Previous studies on the cardiovascular effects of long chain polyunsaturated fatty acids from fish oils have been concerned primarily with the potential antiatherosclerotic effects of these fatty acids. Reported data also include conflicting reports of reduced myocardial necrosis after experimental coronary artery occlusion (17–19), and well-documented modest reductions in blood pressure in normal subjects and in patients with mild hypertension (20–22). Studies by

Table 3. Changes in $[Ca^{2+}]_i$ induced by ouabain

Medium addition	$[Ca^{2+}]_i$, nM		
	0	1 μM	0.1 mM
None	143 ± 11 (8)	294 ± 59 (3)	845 ± 29 (5)
Δ_4Ach	147 ± 11 (7)	329 ± 29 (3)	757 ± 64 (5)
EPA	141 ± 10 (8)	227 ± 2 (3)	214 ± 16 (5)

$[Ca^{2+}]_i$ was measured in neonatal rat cardiac myocytes incubated with ouabain at 0 M, 1 μM, or 0.1 mM by using fura-2. Values are mean ± SEM. Δ_4Ach (5 μM) was added to the incubation medium for 3–5 days. EPA (5 μM) was added to the incubation medium for 3–5 days. $[Ca^{2+}]_i$ measurements were made just prior to and 9–11 min after exposure to ouabain.

Table 4. Inhibition of ^{86}Rb uptake in control and Δ_4Ach- or EPA-enriched myocytes exposed to ouabain, bumetanide, or both

Uptake	^{86}Rb uptake, μmol per mg of protein per min		
	Control	Δ_4Ach	EPA
Total	47.6 ± 1 (11)	46.1 ± 1.4 (11)	47.3 ± 1.6 (11)
Ouabain sensitive	28.9 ± 1.3 (10)	29.3 ± 1.2 (10)	29.6 ± 1.3 (10)
Bumetanide sensitive	4.9 ± 1.5 (11)	4.0 ± 1.2 (11)	4.8 ± 1.7 (11)

Control myocytes or Δ_4Ach- or EPA-enriched myocytes were exposed to 1 mM ouabain, 10 μM bumetanide, or both. The ouabain-sensitive component was determined by subtracting the ouabain-resistant component from the total Rb uptake. The bumetanide-sensitive component was determined by substracting the Rb uptake resistant to the presence of both ouabain and bumetanide from the total Rb uptake. Values are mean ± SEM. Numbers in parentheses are n.

McLennan and Charnock (5, 6) have demonstrated in rats an additional beneficial effect of dietary fish oils, namely, the prevention of fatal ventricular fibrillation induced by ischemia. The recent prospective randomized controlled study reported by Burr *et al.* (7) in men at high risk of recurrent myocardial infarction is consistent with a protective effect against fatal arrhythmias in those advised to eat fish, when the second myocardial infarction did occur. The American Heart Association reports that, of the 500,000 Americans who will die of heart attacks this year, some 300,000 will die before they reach a hospital (23). The great majority of these sudden deaths are caused by ventricular arrhythmias terminating in ventricular fibrillation. Thus potential rhythm-stabilizing action of long chain polyunsaturated fatty acids in fish oils could have noteworthy potential benefits for the public health.

To explore the mechanism(s) of this putative stabilizing antiarrhythmic action of fish oil fatty acids on the heart, we pursued the studies reported here using another arrhythmogenic stimulus, toxic levels of the cardiac glycoside ouabain, in isolated cardiac myocytes prepared from neonatal rat hearts. This preparation has several advantages. (*i*) It allows examination of the effects of fish oil fatty acids that are intrinsic to the affected cell and isolates the effects from possible neurogenic or circulating humoral factors that might cause arrhythmias in the whole animal. (*ii*) The cultured heart cells are robust and survive in culture medium for several

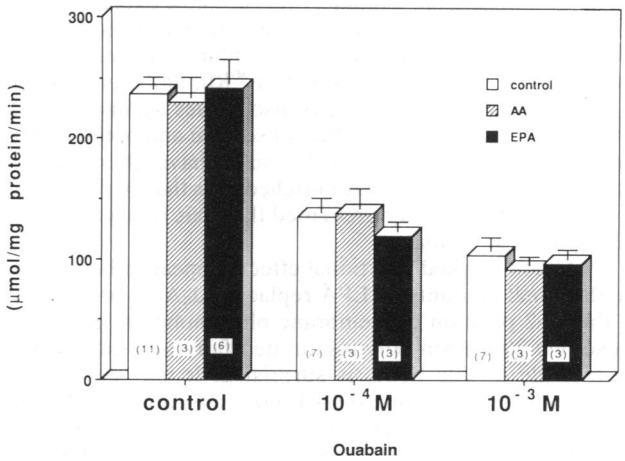

FIG. 2. Effects of ouabain at 0, 0.1, and 1 mM on activity of the Na,K-ATPase in the microsomal fraction of neonatal cardiac myocytes incubated without added fatty acids or with the medium enriched with 5 μM Δ_4Ach or 5 μM EPA for 3–5 days. Na,K-ATPase was measured by the NADH-coupled enzyme method (16). AA, Δ_4Ach.

days, allowing incorporation *in vitro* of specific fatty acids into cell membrane phospholipids. (*iii*) The cells beat spontaneously at a constant rate and amplitude of contraction that can be readily monitored and quantified. (*iv*) The myocytes respond to graded concentrations of cardiac glycosides with an initial positive inotropic response that is followed, at higher glycoside concentrations, by toxic manifestations of rhythm disturbances and contracture.

Clearly, the toxicity in response to 0.1 mM ouabain in these isolated myocytes results in large part from excessive levels of cytosolic calcium, which is prevented by EPA enrichment of cellular phospholipids. Cardiac glycosides have been important in the treatment of patients with heart failure ever since Withering reported the efficacy of foxglove for the treatment of dropsy in 1785. The most worrisome manifestation of toxicity from this class of drugs in humans has been the occurrence of arrhythmias that are potentially lethal. The problem is compounded by the relatively narrow range between the therapeutic and toxic dose and blood levels of this class of medications. Whether the ingestion of fish oils will extend the therapeutic dose range of cardiac glycosides in humans or whether it will simply require higher doses to obtain a therapeutic drug response is not evident from our results.

The generally accepted cellular receptor for ouabain and other cardiac glycosides is the α-subunit of membrane-bound Na,K-ATPase (24); the primary action of cardiac glycosides is to inhibit this enzyme, which is also known as the sodium pump. With inhibition of this enzyme sodium ions accumulate in the cell and (at least at toxic levels of cardiac glycosides) intracellular concentrations of potassium decrease. These ion concentrations changes are associated with partial depolarization of the cell membrane potential. Of particular importance, increased intracellular sodium activity favors the accumulation of calcium ions in the cell via the Na^+–Ca^{2+} antiport system. It is the resulting rise in the intracellular calcium store that is thought to be the principle cause of the inotropic effect of digitalis glycosides on the heart (11). For this reason the idea that EPA incorporated into the membrane phospholipids of the cardiac myocyte somehow prevented ouabain binding or inhibition of the Na,K-ATPase and thus prevented the toxic accumulation of cytosolic calcium seemed a plausible explanation for the lower cytosolic calcium levels observed with EPA. Our results clearly indicate that there was no measurable difference in inhibition of the Na,K-ATPase activity between the EPA-enriched myocytes and the control or Δ_4Ach-enriched myocytes, thus making unlikely this possible explanation for the observed differences in cytosolic calcium levels. The lack of a difference in the bumetanide-inhibitable monovalent cation influx, measured by influx of ^{86}Rb, in the control and Δ_4Ach- and EPA-enriched cells also argues against the possibility that differences in influx of sodium and potassium by the Na^+–K^+–$2Cl^-$ cotransporter might have altered cation homeostasis less in the EPA-enriched cells than in the control cells and in that manner prevented the toxic levels of $[Ca^{2+}]_i$ in response to ouabain.

That such marked functional effects appear to be induced by the small amounts of EPA replacing Δ_4Ach, presumably in the *sn*-2 position of membrane phospholipids, given that these two 20-carbon polyunsaturated fatty acids differ only by a single double bond, is surprising. It may be that the phospholipids containing the EPA are not randomly distributed among all the phospholipids of the myocyte's cell membranes. The studies by Salem *et al.* (25) indicating that EPA- and docosahexaenoic acid-enriched phospholipids may actually be concentrated in the microenvironment of membrane-bound proteins could explain why so small an enrichment with EPA can do so much. Alternatively, the effects of EPA might be due to a reduction in—or competition with—some second messenger derived from Δ_4Ach that modulates calcium homeostasis.

It has been reported that enriching membranes of cardiac myocytes with fish oil fatty acids decreases the activity of the Ca-ATPase of the sarcoplasmic reticulum (26). This would seem to predict an effect on cytosolic calcium levels opposite to that which we observed. Thus, the mechanism by which EPA enrichment of myocyte membranes prevents the toxic levels of $[Ca^{2+}]_i$ after exposure to 0.1 mM ouabain are not yet explained. All pathways involved in calcium homeostasis in the heart, including L- and T-type calcium channels and the Na–Ca exchanger, are worthy of study in relation to the intriguing effects of EPA.

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