Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 9886–9890, October 1994
Physiology

Effects of long-chain polyunsaturated fatty acids on the contraction of neonatal rat cardiac myocytes

(ω6 fatty acid/ω3 fatty acid/cardiomyocyte contraction/automaticity/antiarrhythmics)

JING X. KANG* AND ALEXANDER LEAF*†‡

*Department of Medicine, Harvard Medical School and Massachusetts General Hospital, Boston, MA 02114; and †Brockton/West Roxbury Veterans Affairs Medical Center, West Roxbury, MA 02132

Contributed by Alexander Leaf, July 5, 1994

ABSTRACT **Because of the ability of certain long-chain polyunsaturated fatty acids (PUFAs) to prevent lethal cardiac arrhythmias, we have examined the effects of various long-chain fatty acids on the contraction of spontaneously beating, isolated, neonatal rat cardiac myocytes. The ω3 PUFA from fish oils, eicosapentaenoic acid [EPA; C20:5 (n − 3)] and docosahexaenoic acid [DHA; C22:6 (n − 3)], at 2–10 μM profoundly reduced the contraction rate of the cells without a significant change in the amplitude of the contractions. The fatty acid-induced reduction in the beating rate could be readily reversed by cell perfusion with fatty acid-free bovine serum albumin. Addition of either oxygenase inhibitors or antioxidants did not alter the effect of the fatty acids. Arachidonic acid [AA; C20:4 (n − 6)] produced two different effects on the beating rate, an increase or a decrease, or it produced no change. In the case of the increased or unchanged beating rate in the presence of AA, addition of AA oxygenase inhibitors subsequently reduced the contraction rate. The nonmetabolizable AA analog eicosatetraynoic acid (ETYA) always reduced the beating rate, as did EPA or DHA. Two other PUFAs, linoleic acid [C18:2 (n − 6)] and linolenic acid [C18:3 (n − 3)] also exhibited similar but less potent effects compared with EPA or ETYA. In contrast, neither the monounsaturated fatty acid oleic acid [C18:1 (n − 9)] nor the saturated fatty acids stearic acid (C18:0), myristic acid (C14:0), and lauric acid (C12:0) affected the contraction rate. The inhibitory effect of these PUFAs on the contraction rate was similar to that produced by the class I antiarrhythmic drug lidocaine. The fatty acids that are able to reduce the beating rate, particularly EPA and DHA, could effectively prevent and terminate lethal tachyarrhythmias (contracture/fibrillation) induced by high extracellular calcium concentrations or ouabain. These results suggest that free PUFAs can suppress the automaticity of cardiac contraction and thereby exert their antiarrhythmic effects.**

It has become apparent that long-chain polyunsaturated fatty acids (PUFAs) can affect the functions of cells in many ways. Through their active cyclooxygenase, lipoxygenase, and epoxygenase metabolites they create potent cellular messengers. But as free fatty acids they also can exert regulatory functions. Since PUFAs are an integral part of the phospholipids of cellular membranes, it is often not certain whether they exert their effects on cell functions after enzymatic conversion to an active metabolite, after covalent incorporation into phospholipid molecules, as free fatty acids interdigitated between the acyl chains of the membrane bilayers, or while directly bound to membrane proteins.

A beneficial effect of long-chain ω3 fatty acids from fish oils on cardiovascular disease has been well recognized (for review see ref. 1). Recent studies indicate a role for the fish oil fatty acids in the prevention of fatal ventricular arrhythmias. As Charnock, McLennan, and co-workers (2–4) have found from feeding experiments in rats and marmosets, Billman *et al.* (5) have shown that an intravenous infusion of an emulsion composed largely of eicosapentaenoic acid [EPA; C20:5 (n − 6)] and docosahexaenoic acid [DHA; C22:6 (n − 3)] can prevent ischemia-induced ventricular fibrillation in conscious, prepared dogs. Our earlier studies also indicate that EPA and DHA at 2–5 μM can prevent toxic arrhythmias due to ouabain (0.1 mM), and this protective action seems associated with inhibition of the cytosolic free calcium overload that characterizes ouabain toxicity (6, 7). However, questions as to what is the primary effect of the fish oil fatty acids on the contraction of cardiac myocytes, whether the effect of the fatty acids depends on their incorporation into membrane phospholipid or their metabolism, and whether different classes of fatty acid exert the same effect remain unanswered. This study demonstrates that the rate of contraction of isolated neonatal rat cardiac myocytes can be significantly reduced by both n − 3 and n − 6 free (unesterified) PUFAs, including linolenic [C18:3 (n − 3)], linoleic acid [C18:2 (n − 6)], and, under certain conditions, arachidonic acid [AA; C20:4 (n − 6)] as well as EPA and DHA, but not by monounsaturated oleic acid [C18:1 (n − 9)] or the saturated acids tested, including stearic acid (C18:0), myristic acid (C14:0), and lauric acid (C12:0).

METHODS AND MATERIALS

Cell Culture. Cardiac myocytes were isolated from 1-day-old rats by using the Neonatal Cardiomyocyte Isolation System (Worthington). This system, utilizing purified enzyme preparations, provides a reliable and consistent cell isolation method. Isolation was performed according to the manufacturer's recommendations. Cells were cultured at 37°C in an atmosphere with 5% CO_2 and 98% relative humidity in a tissue culture incubator (model 3123; Forma Scientific, Marietta, OH). The culture medium was changed every other day. After 48 hr in culture, cells exhibited regular spontaneous contractions. Cells were used for experiments after 3–5 days of culture.

Measurement of Contractility. Changes in amplitude of contraction and beating rate of cultured cardiomyocytes were determined by using a phase-contrast microscope and video monitor edge-detector as described (Crescent Electronics, Salt Lake City). A glass coverslip with attached cultured myocytes was continuously superfused during contractility measurements with a Hepes/saline solution (in mM: 140

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: AA, arachidonic acid; BSA, bovine serum albumin; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ETYA, eicosatetraynoic acid; PUFA, polyunsaturated fatty acid.

‡To whom reprint requests should be addressed at: Massachusetts General Hospital, East 4th Floor, 149 13th Street, Charlestown, MA 02129.

NaCl, 5 KCl, 1.0 $MgCl_2$, 1.2 $CaCl_2$, 1.0 Na_2HPO_4, 5.0 Hepes, 10 glucose; pH was adjusted to 7.4 with NaOH). The flow rate was 20 ml/hr. The chamber temperature was maintained at 32°C. After approximately a 10-min equilibration period, fatty acid was added to the perfusion fluid to a final concentration of 5–10 μM. Changes in amplitude and rate of cell contractions were used to assess the effects of various fatty acids on cell contractions.

Materials. Fatty acids, eicosatetraynoic acid (ETYA), phytanic acid, cholesterol, indomethacin, and fatty acid-free bovine serum albumin (BSA) were obtained from Sigma. BW 755c was kindly provided by M. D. Cooke (Wellcome Foundation). Fatty acids were dissolved weekly in ethanol at 10 mM and stored under a nitrogen atmosphere at −20°C. The final concentration of ethanol was negligible and had no effect on myocyte contraction.

RESULTS

Effects of Fish Oil Fatty Acids on Contractions of Myocytes. Perfusion of the cultured myocytes with medium containing 5–10 μM EPA or DHA reduced the contraction rate of the cells by 50–80% after 3 min of perfusion without a significant change in the amplitude of contraction of the cells (Fig. 1 *A* and *B*). To test whether this effect of EPA and DHA on the contraction rate was reversible, fatty acid-free BSA (2.0 mg/ml or 30 μM) was added to the perfusion fluid. Fig. 1 shows that perfusion of the myocytes with BSA resulted in a rapid reversal of the beating rate (within 2 min). In this preparation BSA alone at 2.0 mg/ml had no effect on myocyte contractions. The reduction of the contraction rate by the fatty acids is similar to that produced by lidocaine (20 μM), a typical class I antiarrhythmic drug (Fig. 1*C*).

To test whether the effect of EPA or DHA relies on its metabolites, either indomethacin (10–20 μM; a cyclooxygenase inhibitor) and BW 755c (20 μM; a lipoxygenase inhibitor) or butylated hydroxytoluene (BHT; 0.005%) and vitamin E (0.5 unit/ml) (antioxidants) were added together to the perfusion fluid. Neither the enzyme inhibitors nor antioxidants altered EPA-induced reductions in beating rate. In addition, ETYA, a potent inhibitor of cyclooxygenase, lipoxygenase, and expoxygenase, did not change the effect of EPA or DHA.

To test whether the PUFA-induced reduction in beating rate has a protective effect against high extracellular Ca^{2+} ($[Ca^{2+}]_o$) or ouabain-induced contractures and fibrillations of the cardiomyocyte, Ca^{2+} (7–10 μM) or ouabain (0.1 mM) was added to the perfusion medium before and after perfusion of the cells with EPA or DHA. Fig. 2 *A* and *B* shows that high $[Ca^{2+}]_o$ or 0.1 mM ouabain failed to induce contractures and fibrillation after perfusion with EPA (5 μM), in keeping with our earlier findings (7). In addition to illustrating this preventive effect, Fig. 2*C* shows that EPA could also terminate ouabain plus Ca^{2+}-induced contractures and fibrillation, and extracting the free fatty acid with BSA reestablished contractures and fibrillation.

Effects of AA on Contractions of Myocytes. Perfusion of the myocytes with 5–10 μM AA produced two different effects on the rate of contractions, increase or decrease, or it produced no change (Table 1; Fig. 3 *A*, *B*, and *C*). In 16 of 48 measurements, an increase in both the beating rate of contractions of the myocyte and, in some cases, even contracture and fibrillation of the cell was observed following perfusion with AA (Fig. 3*A*). Eighteen of 48 measurements showed a reduction in the beating rate with slight increases in amplitude of contractions within 3–5 min after perfusion with AA (similar to the effect observed with EPA or DHA) (Fig. 3*C*). Fourteen of the 48 measurements exhibited no significant change in beating rate (Fig. 3*B*).

Interestingly, whether the contraction rate was increased or unchanged following addition of AA alone to the perfusion medium, the rate could be slowed within 5 min to 30–50% of the control pre-AA rate when a combination of indomethacin (20 μM; a cyclooxygenase inhibitor) and BW 755c (20 μM; a lipoxygenase inhibitor) was added in the presence of AA (5–10 μM) to the perfusion fluid (Fig. 3 *A* and *B*). Indomethacin (20 μM) plus BW 755c (20 μM) alone did not reduce the beating rate significantly within 10 min, unless AA was subsequently added to the perfusion solution (in which case AA always reduced the contraction rate). Moreover, perfusion of myocytes with ETYA (5 μM), a nonmetabolizable analog of AA and a potent inhibitor of the cyclooxygenase, lipoxygenase, and expoxygenase enzymatic pathways, always reduced the beating rate within 2–3 min. The AA- or ETYA-induced reductions in contraction rate could be quickly reversed by perfusion with BSA (2.0 mg/ml).

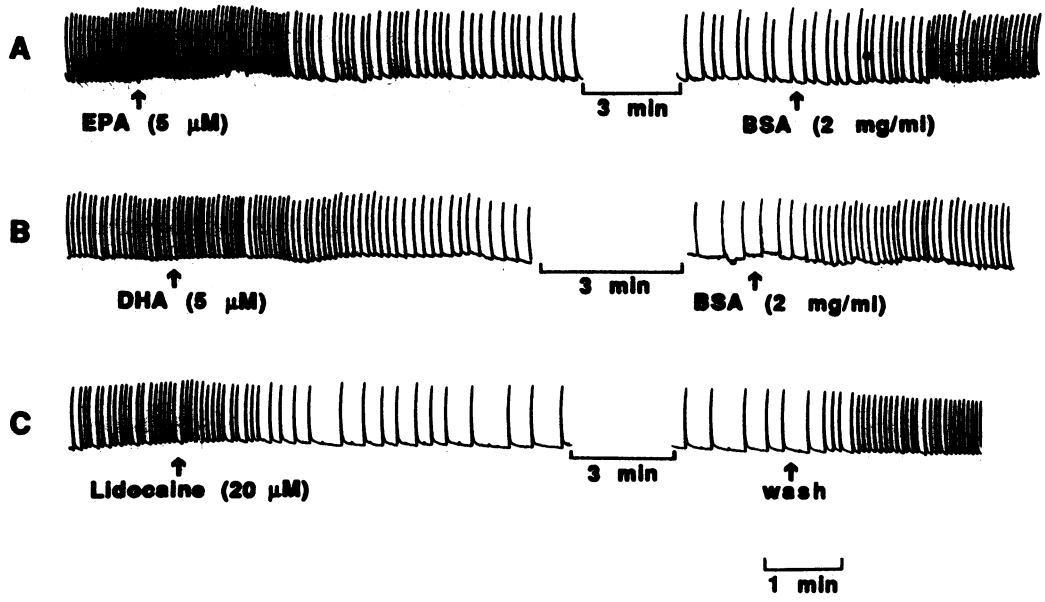

FIG. 1. Effects of EPA and DHA on the contraction of isolated neonatal rat cardiomyocytes. Perfusion of the myocytes with 5 μM EPA (*A*) or 5 μM DHA (*B*) reduced the beating rate by 50% within 2 min, and addition of BSA to the perfusion solution quickly reversed the effect. Tracing *C* shows a similar effect of lidocaine (20 μM) on the contraction of the cardiac myocytes.

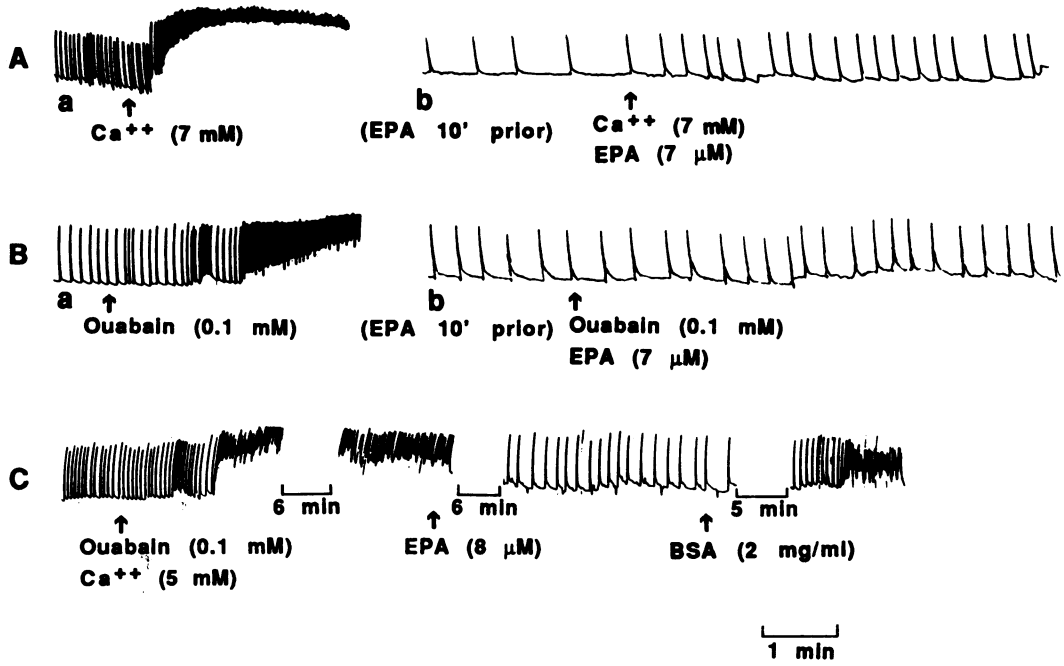

FIG. 2. Tracings show prevention and termination of arrhythmia by EPA. Perfusion of the myocytes with a solution containing 7 μM Ca^{2+} (*A*, *a*) or 0.1 mM ouabain (*B*, *a*) induced contracture and fibrillation before perfusion with EPA. Washing the cells with medium (Ca^{2+} = 1.2 mM) returned the fibrillations to the original beating rate (not shown). Then the cells were perfused with medium containing 7 μM EPA. After 5–8 min, when the beating rate was slowed, addition of 7 mM Ca^{2+} (*A*, *b*) or 0.1 mM ouabain (*B*, *b*) failed to induce contracture or fibrillation in the same cells. The slow beating rates were subsequently returned to the original rates by perfusion with BSA (not shown). (*C*) Alternatively, after induction of fibrillation by ouabain (0.1 mM) plus Ca^{2+} (5 mM), addition of EPA (8 μM) terminated the fibrillation and led to slow beating, and subsequent addition of BSA (2 mg/ml), still in the presence of ouabain and high external Ca^{2+} concentration, reinstated fibrillation.

To test whether EPA can attenuate AA-induced effects, EPA (5–10 μM) was added to perfusion solution when the AA-induced increase in the rate of contraction or contracture of the myocytes occurred. Fig. 3*E* shows that AA-induced contracture of the myocytes was abolished and subsequently the beating rate was slowed within 6–10 min after addition of EPA, indicating an opposing effect of EPA against AA stimulation in this condition.

Effects of Other Fatty Acids on Contraction Rate of Myocytes. To test whether other PUFAs, monounsaturated fatty acids, or saturated fatty acids have similar effects on myocyte contractions, myocytes were perfused with 10 μM linoleic acid [C18:2 (*n* − 6)], linolenic acid [C18:3 (*n* − 3)], oleic acid [C18:1 (*n* − 9)], stearic acid (C18:0), myristic acid (C14:0), or lauric acid (C12:0). As summarized in Table 1, both PUFAs exhibited inhibitory effects on the beating rate similar to EPA and DHA, but their effects were less potent (reduction in beating rate by 30–50% within 7 min) compared with DHA or EPA. Their effects could also be reversed by perfusion with 0.2% BSA. In contrast, neither the monounsaturated fatty acid C18:1 (*n* − 9) nor the saturated fatty acids C18:0, C14:0, and C12:0 affected the contraction rate. Furthermore, EPA ethyl ester, a non-free acid form of EPA, also did not affect myocyte contractions, indicating that the free fatty acid is essential for this effect.

Table 1. Effects of various fatty acids on the beating rate of isolated neonatal rat cardiac myocytes

Fatty acid	Effect	No./no. tested
EPA	−	46/46
DHA	−	32/32
AA	−	18/48
	+	16/48
	±	14/48
ETYA	−	18/18
AA + inhibitors	−	28/30
C18:3 (*n* − 3)	−	5/5
C18:2 (*n* − 6)	−	4/4
C18:1 (*n* − 9)	±	4/4
C18:0	±	6/6
C14:0	±	3/3
C12:0	±	3/3
EPA ethyl ester	±	3/3
Phytanic acid	±	9/12
	−	3/12
Cholesterol	±	5/5

Effects: −, inhibitory (reduction in beating rate); +, stimulatory (increase in beating rate); and ±, none (no change in beating rate).

To assess whether the effects of PUFAs on myocyte contraction are due to a possible change in membrane disorder, phytanic acid (8) and cholesterol, which are known to increase and decrease membrane disorder, respectively, were used to perfuse the myocytes. In 9 of 12 measurements, phytanic acid (10 μM) failed to reduce the beating rate. However, when EPA was subsequently added to the medium, the beating rate was slowed within 3–5 min. The other three measurements with phytanic acid showed a slight decrease of the beating rate which could not be reversed by BSA. Cholesterol (10 μM) apparently did not affect myocyte contraction, similar to the finding with the saturated fatty acids.

DISCUSSION

The present results indicate that common dietary PUFAs of both the ω3 and ω6 classes, in addition to EPA and DHA, can modulate the contraction rate of spontaneously beating, isolated, neonatal cardiac myocytes. Monounsaturated oleic and saturated stearic, myristic, and lauric acids had no such effect.

The reduction of the contraction rate by PUFAs indicates an effect of the fatty acids on excitability (automaticity) of cardiac myocytes. Since depression of automaticity is a

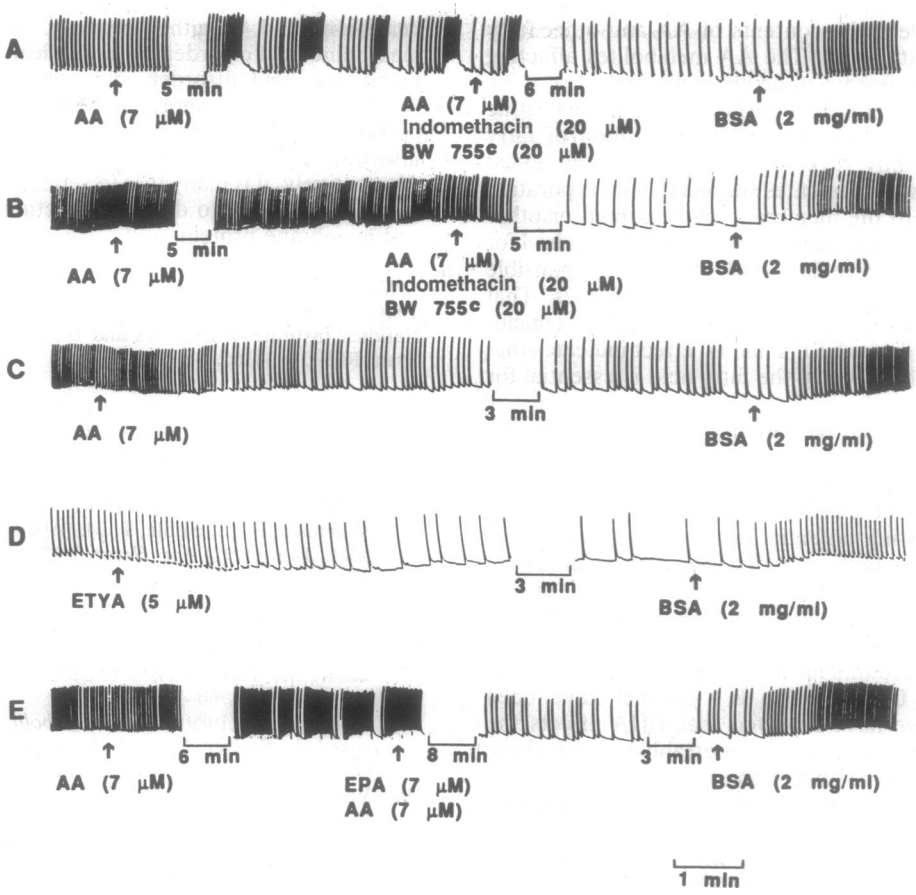

FIG. 3. Effects of AA and ETYA on the contraction of isolated neonatal rat cardiac myocytes. (*A*) Perfusion of the myocytes with 7 μM AA induced contracture of the cell. Addition of indomethacin (20 μM) plus BW 755c (20 μM) abolished the contracture within 5 min and subsequently induced a slow beating rate, which could be returned to normal by BSA. (*B*) Initial perfusion with 7 μM AA did not change the beating rate. Addition of indomethacin (20 μM) and BW 755c (20 μM) resulted in a reduction of the beating rate, which could be reversed by BSA. (*C*) Perfusion with AA alone slowed the beating of the myocytes. Addition of BSA reversed the beating rate. (*D*) ETYA (5 μM) significantly reduced the beating rate within 3–4 min. Perfusion with BSA (2 mg/ml) reversed this effect. (*E*) AA (7 μM) induced contraction of the myocytes. Addition of EPA (7 μM) abolished the AA-induced contracture and subsequently slowed the beating. Perfusion with BSA reestablished the original beating rate.

critical property of class I antiarrhythmic drugs such as lidocaine, free PUFAs could have beneficial effects on arrhythmias due to increased automaticity, such as ischemia-induced ventricular tachycardia and fibrillation. As shown in Fig. 2, perfusion of the myocytes with EPA or DHA (5–7 μM) could prevent and terminate high extracellular Ca^{2+} ($[Ca^{2+}]_o$) and ouabain-induced fibrillations, due to overload of cytosolic Ca^{2+}. This protective effect of the fatty acids may be attributable, at least in part, to the fatty acid-induced reduction of the beating rate. The present observation is in agreement with our previous finding that an intravenous infusion of the ω3 fatty acids reduced heart rate and prevented ischemia-induced ventricular fibrillation in dogs (5).

AA proved to be a special case in that in almost equal thirds in these observations it slowed, accelerated, or did not alter the rate of contraction of the cardiac myocytes. This encompasses all possibilities and perhaps explains some of the existing confusion and contradictory findings in previous studies (reviewed in ref. 9), including our own (6, 7). Another source of possible confusion may be the high concentrations of free fatty acids often used when testing effects of the fatty acids on cell functions. High free fatty acid concentrations have been repeatedly shown to have nonspecific toxic effects on cells, damaging cell membranes and mitochondrial function. Concentrations of free fatty acids of 10 μM have been estimated to be the upper limit occurring normally in cells (10), and concentrations of <10 μM in the absence of albumin should be used when studying possible physiologic actions of fatty acids.

This study was not performed to identify specific oxidized metabolites of the long-chain fatty acids that might change the contraction rate of the myocytes. It is of interest, however, when addition of AA to the myocytes either increased the rate of contraction or left it unchanged, that agents which block formation of active cell messengers from AA invariably slowed the beating rate. On the other hand, EPA, DHA, and ETYA, in all instances tested, slowed the rate of contraction of the myocytes. This results in the interesting inference that of these fatty acids, the action of the free acid alone is to slow the contraction rate, particularly since ETYA is incapable of producing metabolites analogous to those derived from AA, and it unfailingly only reduced the rate. Also any metabolites being synthesized from EPA or DHA under the conditions of this study must be either physiologically inactive or inhibitory of the contraction rate, since, unlike AA, they also only slowed the contraction rate. C18:2 ($n - 6$) and C18:3 ($n - 3$) also only reduced the rate of contractions but more slowly and to a lesser degree than EPA, DHA, or ETYA. Under the conditions of these experiments it seems unlikely that C18:2 ($n - 6$) could be converted to AA to reproduce the variety of AA effects on the myocytes. It seems that the nature of the effect of AA on myocyte contraction is determined by the status of AA metabolism in the myocytes. Thus, the outcome of supplementations of the cell with AA depends on the balance between the effects of free AA and AA metabolites

or the balance between the contents of AA and other fatty acids such as ω3 fatty acids. The AA metabolites affecting myocyte contraction remain to be characterized.

The findings that the effects of the fatty acids on the beating rate took place within 2–5 min and that addition of fatty acid-free BSA promptly reversed the effects of the added fatty acids on the contraction rates suggest that incorporation of the fatty acids into the membrane phospholipid or other covalent linking to membrane components is not required for their action and that the free fatty acid is the form responsible for the slowing of the beating rate of the myocytes. That ETYA only slowed the rate strongly supports this conclusion. That the ethyl ester of EPA had no effect indicates that the intact carboxyl function of the fatty acid is essential for its effects.

The precise mechanism by which free PUFAs slow the contractions of the cardiomyocytes remains unknown. Decrease in excitability of the cells may result from elevation of threshold (a more positive membrane potential of the threshold), increase in resting potential (a more negative resting potential), or decrease in the rate of depolarization of resting potential (11). Changes in activity of Ca^{2+}, Na^{+}, or K^{+} channels may be responsible for the above effects (11). Thus further study to identify the ion channel(s), the effect of which specifically accounts for the PUFA effect, is needed. Though effects of DHA on the K^{+} channel have been reported (12) and we have reported that DHA affects the L-type Ca^{2+} channel (10), it is not yet established that these actions are primary to the effects on exitability shown here. How these fatty acids exert their effect on ion channels or other proteins also needs to be determined. It is possible that partition of free fatty acids into membrane lipid bilayer leads to changes in membrane properties which affect the functions of membrane proteins. However, it seems unlikely that the effects of PUFAs are due to change in the membrane lipid order as a result of fatty acid partition, because addition to the perfusion fluid of either phytanic acid, which increases membrane lipid disorder (8), or cholesterol, which decreases membrane lipid disorder, did not significantly affect the beating rate. Furthermore, increasing the temperature of the perfusion fluid, which may also increase membrane lipid disorder, increased rather than decreased the beating rate. Alternatively, it is likely that the effect of PUFAs on myocyte contraction is due to direct interactions between fatty acid and membrane proteins.

We are grateful to Caren Kachoris and Xia Mei Liu for their assistance with the cell culture. This study was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grant R01-DK 38165.

1. Leaf, A. (1992) *Nutr. Rev.* **50,** 150–154.
2. McLennan, P. L., Abeywardena, M. Y. & Charnock, J. S. (1985) *Can. J. Physiol. Pharmacol.* **63,** 1441–1447.
3. McLennan, P. L., Abeywardena, M. Y. & Charnock, J. S. (1988) *Am. Heart J.* **16,** 709–717.
4. Mclennan, P. L., Bridle, T. M., Abeywardena, M. Y. & Charnock, J. S. (1992) *Am. Heart J.* **123,** 1555–1561.
5. Billman, G. E., Hallaq, H. & Leaf, A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4427–4430.
6. Hallaq, H., Thomas, A. S. & Leaf, A. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 7834–7838.
7. Hallaq, H., Thomas, A. S. & Leaf, A. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1760–1764.
8. Steinberg, D. (1989) in *The Metabolic Basis of Inherited Disease*, eds. Scriver, C. V., Beaudet, A. L., Sly, W. S. & Valle, D. (McGraw-Hill, New York), 6th Ed., pp. 1533–1550.
9. Van Der Vusse, G. J., Glatz, J. F. C., Stam, H. C. G. & Reneman, R. S. (1992) *Physiol. Rev.* **72,** 881–940.
10. Pepe, S., Bogdanov, K., Hallaq, H., Spurgeon, H., Leaf, A. & Lakatta, E. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 8832–8836.
11. Berne, R. M. & Levy, M. N., eds. (1993) in *Physiology* (Mosby Year Book, St. Louis), 3rd Ed., pp. 364–396.
12. Honore, E., Barhanin, J., Attali, B., Lesage, F. & Lazdunski, M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 1937–1944.