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Physiology

Free, long-chain, polyunsaturated fatty acids reduce membrane electrical excitability in neonatal rat cardiac myocytes

(action potential/threshold/automaticity/ω3 and ω6 fatty acids/antiarrhythmics)

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ABSTRACT **Because previous studies showed that polyunsaturated fatty acids can reduce the contraction rate of spontaneously beating heart cells and have antiarrhythmic effects, we examined the effects of the fatty acids on the electrophysiology of the cardiac cycle in isolated neonatal rat cardiac myocytes. Exposure of cardiomyocytes to 10 μM eicosapentaenoic acid for 2–5 min markedly increased the strength of the depolarizing current required to elicit an action potential (from 18.0 ± 2.4 pA to 26.8 ± 2.7 pA, $P < 0.01$) and the cycle length of excitability (from 525 ms to 1225 ms, $\Delta = 700 \pm 212$, $P < 0.05$). These changes were due to an increase in the threshold for action potential (from −52 mV to −43 mV, $\Delta = 9 \pm 3$, $P < 0.05$) and a more negative resting membrane potential (from −52 mV to −57 mV, $\Delta = 5 \pm 1$, $P < 0.05$). There was a progressive prolongation of intervals between spontaneous action potentials and a slowed rate of phase 4 depolarization. Other polyunsaturated fatty acids—including docosahexaenoic acid, linolenic acid, linoleic acid, arachidonic acid, and its nonmetabolizable analog eicosatetraynoic acid, but neither the monounsaturated oleic acid nor the saturated stearic acid—had similar effects. The effects of the fatty acids could be reversed by washing with fatty acid-free bovine serum albumin. These results show that free polyunsaturated fatty acids can reduce membrane electrical excitability of heart cells and provide an electrophysiological basis for the antiarrhythmic effects of these fatty acids.**

Recent studies have shown a role for ω3 fatty acids in the prevention of fatal ventricular arrhythmias. Clinical trials in humans demonstrated significant reduction in the incidence of sudden death from coronary heart disease in subjects whose diets contained long-chain ω3 fatty acids (1, 2). McLennan *et al.* (3, 4) found that a diet high in fish oil, in contrast to saturated fat, prevented ventricular fibrillation induced by coronary artery ligation in rats and increased the electrical ventricular fibrillation thresholds in marmosets (5). Billman *et al.* (6) have shown that an i.v. infusion of an emulsion composed largely of eicosapentaenoic acid (EPA, 20:5ω3) and docosahexaenoic acid (DHA, 22:6ω3) can prevent ischemia-induced ventricular fibrillation in conscious, prepared dogs (6). Our previous studies also indicate that free long-chain polyunsaturated fatty acids (puFAs), especially fish oil ω3 fatty acids (EPA and DHA), at 5–10 μM can markedly reduce the contraction rate of isolated, spontaneously beating cardiomyocytes and prevent or terminate the tachyarrhythmias and fibrillation induced by ouabain (7–9), high extracellular calcium (9), β-adrenergic agonist (10), or lysophosphatidylcholine and acylcarnitine (unpublished data). However, the mechanisms underlying the antiarrhythmic effect of the fatty acids have not been elucidated (at the cellular level). We have examined the effects of various fatty acids on membrane electrophysiology in neonatal rat cardiac myocytes. We report here that free puFAs, but neither monounsaturated nor saturated fatty acids, at 5–10 μM significantly reduce membrane electrical excitability by increasing the threshold for action potential (more positive), the resting-membrane potential (more negative), and the refractory-period duration in the cardiac myocytes.

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METHODS AND MATERIALS

Cell Culture. Cardiac myocytes were isolated from 1-day-old rats using a neonatal cardiomyocyte isolation system (Worthington). This system, with purified enzyme preparations, provides a reliable and consistent cell-isolation method. Cells were cultured at 37°C in air/5% CO_2 (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 3–6 days of culture.

Measurement of Membrane Action Potential. All experiments were done at room temperature (22°C). Recordings were made with glass microelectrodes (2–5 MΩ, World Precision Instruments, Sarasota, FL) and a Dagan Instruments (Minneapolis) 3900 patch-clamp amplifier. Data were stored in the hard disk of a personal computer and analyzed with PCLAMP 5.5.1 (Axon Instruments, Burlingame, CA). The bathing solution consisted of 140 mM NaCl, 5.0 mM KCl, 1.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, and 10 mM Hepes, at pH 7.4. The pipette solution contained 140 mM KCl, 2.0 mM $MgCl_2$, 1.0 mM $CaCl_2$, 5.0 mM MgATP, 10 mM NaCl, 10 mM Hepes, and 10 mM EGTA, at pH 7.4.

The experimental protocol began 5 min after breaking into the cell. In the standard protocol, the holding potential was set to ≈−70 mV. To initiate an action potential, the cells were stimulated with either a fixed (constant) depolarizing impulse (30 pA with a 15-ms duration) or a series of depolarization pulses (20 ms) of 3- or 4-pA increment from the holding potential. The membrane potential at which a further minimal depolarization elicited an action potential was regarded as the threshold for the action potential. Cycle lengths of the excitability were measured by giving the cells a pair of superthreshold electrical stimuli (40 pA/20 ms) with various intervals from 500 to 1500 ms in 100-ms increment. The frequency of stimulus for all protocols was 0.1–0.05 Hz. In some case, spontaneous action potentials were detected with the zero current-clamp method.

Materials. A neonatal cardiomyocyte isolation system kit was purchased from Worthington. Fatty acids, eicosatetray-

Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ETYA, eicosatetraynoic acid; puFA, polyunsaturated fatty acid.

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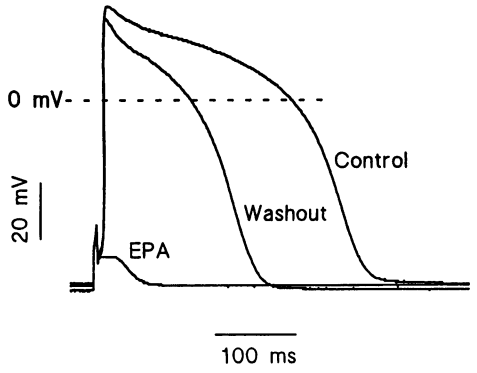

FIG. 1. Effect of EPA on generation of action potentials in response to a fixed depolarizing impulse. Injection of a depolarizing current (30 pA/15 ms at 0.05 Hz) initiated typical action potentials before EPA addition. After exposure to 10 μM EPA the same depolarizing current failed to elicit an action potential. Washing with bovine serum albumin at 2 mg/ml regenerated the action potential.

noic acid (ETYA), and fatty acid-free bovine serum albumin were from Sigma. Fatty acids were dissolved weekly in ethanol at 10 mM concentration and stored under a nitrogen atmosphere at −20°C. The final concentration of ethanol was negligible and had no effect on the parameters of the myocyte action potential.

Statistics. All results are presented as means ± SEMs. Comparisons were made by paired Student's *t* test; the level of statistical significance was considered at $P < 0.05$.

RESULTS

Exposure of isolated rat ventricular myocytes to 5–10 μM EPA suppressed the excitability of the cells. Fig. 1 shows a representative recording of the effect of EPA on the generation of action potentials in response to given depolarizing current pulses. Before EPA addition, stimulation of the cells with constant depolarizing current pulses (30 pA for 15 ms) induced typical action potentials. When EPA (5–10 μM) was added to the bath solution, within 1- to 2-min application of the same depolarizing current, pulses failed to elicit action potentials. However, subsequent washing with solution containing fatty acid-free bovine serum albumin (2 mg/ml) quickly reinstated action potentials during continuous stimulation with the same depolarizing current pulses. To determine the EPA-induced changes in the threshold for the action potential, myocytes were stimulated with a series of depolarizing current pulses in 3- or 4-pA increment before and after 10 μM EPA exposure. Fig. 2 shows that the stimulation strengths required to initiate action potentials before and after EPA exposure were 18.0 ± 2.4 pA and 26.8 ± 2.7 pA ($P < 0.01$), respectively. Analysis of the data revealed that the increased stimulation strength after EPA exposure was due to an increase in both action-potential thresholds from −52 mV to −43 mV ($P < 0.05$) and the resting membrane potentials from −52 mV to −57 mV ($P < 0.05$). These effects of EPA could be reversed by washing with bovine serum albumin (2 mg/ml) added to the superfusion medium.

To determine the effect of EPA on the cycle length of excitability that could reflect the refractory period, myocytes were stimulated with a pair of superthreshold depolarizing current pulses at different intervals. Fig. 3 shows that the cycle lengths were remarkably prolonged (from 525 ms to 1225 ms, $\Delta = 700 \pm 212$, $P < 0.05$) as a result of exposure to EPA (10 μM). Fig. 4 shows the spontaneous generation of action potentials before and after 10 μM EPA exposure. Three minutes after EPA addition, the intervals between action potentials were significantly prolonged, resulting in ≈50% reduction in the frequency of action potentials. This reduction was due to alterations in the same electrical parameters affected by EPA in the stimulated myocytes—namely, hyperpolarization of the resting membrane potential (in this case with $n = 7$ from −58 ± 2 mV to −62 ± 3 mV), rise in the threshold voltage (from −45 ± 3 mV to −40 ± 3 mV), and prolongation of the refractory time, which in these spontaneously beating myocytes results from a decreased slope of the phase 4 depolarization. The effect of EPA on the frequency of action potentials is consistent with its effect on the contraction rate observed in our previous study (9).

The EPA-induced changes in properties of the action potential are summarized in Table 1. It is noted that the main effects were on action-potential threshold, resting- and holding-membrane potentials, and action-potential duration, while the action-potential amplitude and maximum rate of phase 0 depolarization were not significantly affected.

The effects of other fatty acids—including DHA (22:6ω3), arachidonic acid (20:4ω6), ETYA (a nonmetabolizable acetylenic arachidonic acid analog), linoleic acid (18:2ω6), linolenic acid (18:3ω3), oleic acid (18:1ω9), and stearic acid (18:0)—on the electrical parameters of the contraction cycle were also tested under the same conditions as for EPA. The results (data not shown) indicates that the puFAs, DHA, arachidonic acid, ETYA, linolenic acid, and linoleic acid, but neither the monounsaturated oleic acid nor the saturated stearic acid ($n = 3$, for each fatty acid tested), have effects similar to EPA.

DISCUSSION

The present study demonstrates that the long-chain puFAs but neither the monounsaturated nor the saturated fatty acids, can

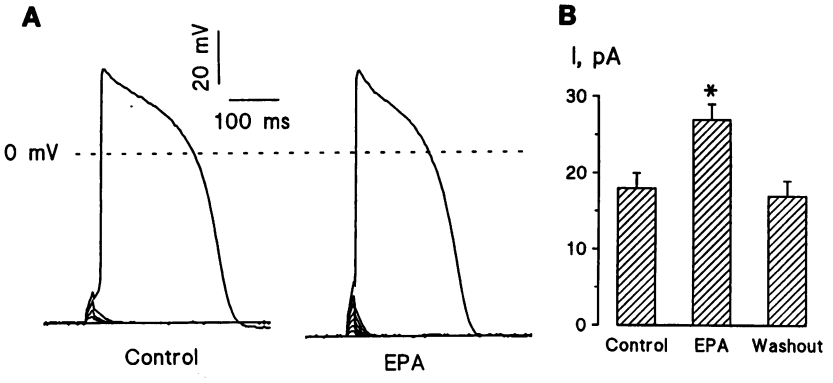

FIG. 2. Effect of EPA on holding voltage at constant imposed current, threshold voltage, and current required to attain action-potential threshold. Holding potential was initially set at ≈−70 mV, and cells were stimulated with a series of depolarization pulses in 3- or 4-pA increment at 10-s intervals. (*A*) Recordings showing the holding transmembrane potentials, the threshold potentials, and the currents required to elicit an action potential in a cell before (*Left*) and after (*Right*) exposure to 10 μM EPA. Washing with bovine serum albumin reversed the effect of EPA (recording not shown). (*B*) Mean values ± SEM ($n = 8$) of the currents required to elicit an action potential before EPA exposure, after EPA exposure, and with EPA washout from eight individual experiments. *, $P < 0.01$.

significantly but reversibly increase the action-potential threshold, the resting membrane potential, and the cycle length, thereby reducing membrane electrical excitability in isolated neonatal rat cardiac myocytes. This result provides an electrophysiological basis for the antiarrhythmic effects of free puFAs.

Our previous study (9) showed that perfusion of isolated neonatal rat cardiac myocytes with 5–10 μM EPA or other puFAs remarkably reduced the contraction rate of the cells. Similarly, i.v. infusion of fish oil emulsion significantly reduced heart rate in dogs (6). Furthermore, McLennan *et al.* (5) found that feeding marmoset monkeys with diet supplemented with puFA significantly elevated the ventricular fibrillation threshold. These observations can be explained by the present finding that puFAs, particularly fish-oil ω3 fatty acids, suppress myocardial membrane electrical excitability. This property of the fatty acids provides a valuable anti-tachyarrhythmic action, which supports the protective effects of ω3 fatty acids on fatal arrhythmias, as noted by several previous studies (3–10).

Change in excitability can be an important factor in the generation or termination of arrhythmias. The excitability of

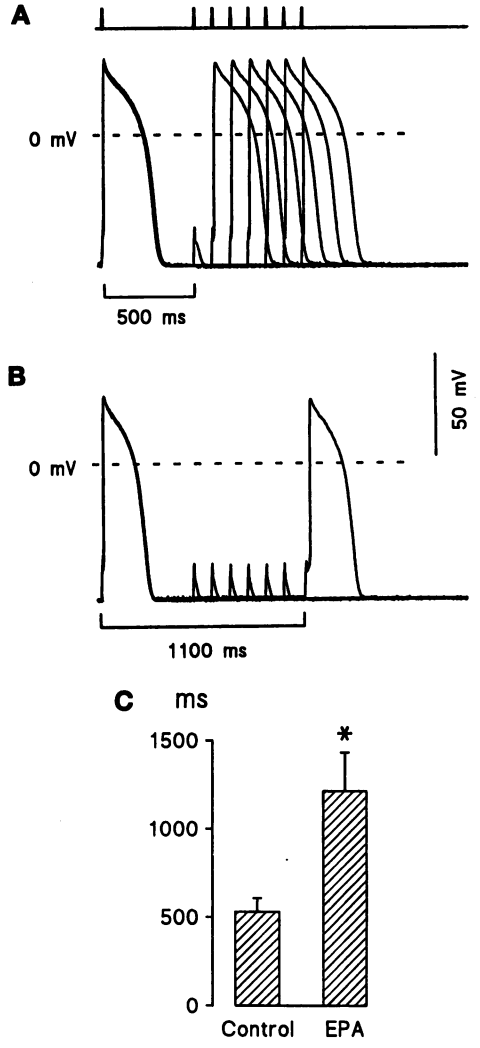

FIG. 3. The effect of EPA on the cycle length of excitability. Cells were given a pair of superthreshold electrical stimuli at 0.1 Hz (necessary to elicit action potential in the presence of EPA) with various intervals from 500 to 1500 ms in 100-ms increments. Bars at top indicate the time intervals between two stimuli. (*A*) Recording showing the cycle length (600 ms) before 10 μM EPA exposure. (*B*) Recording showing cycle length (1100 ms) 3 min after EPA exposure. The cycle length before and after EPA exposure from four individual experiments was 525 ms and 1225 ms (Δ = 700 ± 212), respectively. *, $P < 0.05$.

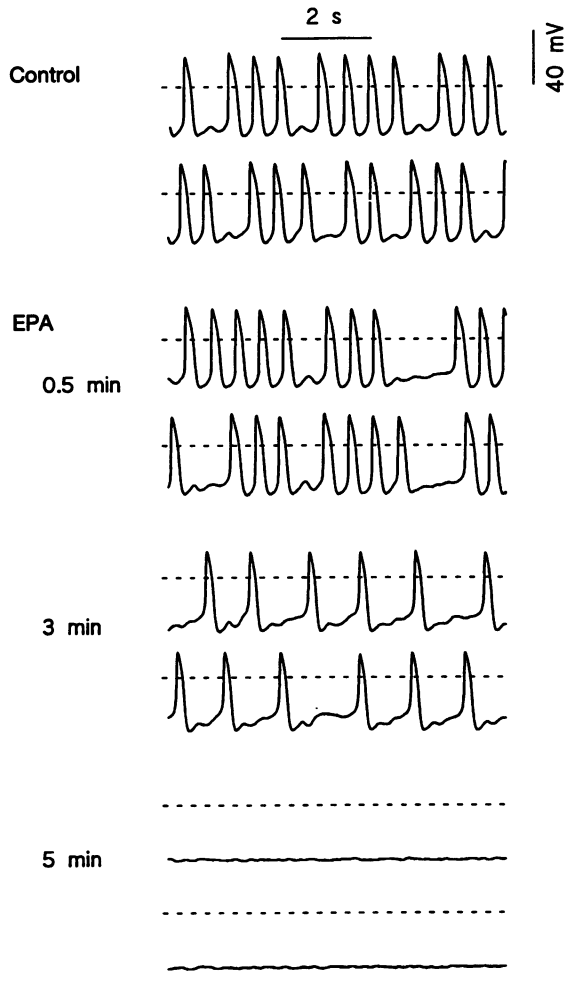

FIG. 4. Patch-clamp recording showing the action potentials of spontaneously beating neonatal rat cardiac myocytes before and after 10 μM EPA exposure. Addition of EPA to the bath medium gradually prolonged the action-potential intervals with time. - - -, Zero-voltage level.

cardiomyocytes may be altered by a change in the threshold potential, the resting membrane potential, or the rate of depolarization during phase 4 (11). It has been reported that during acute myocardial ischemia both the threshold potential and the magnitude of the resting potential are reduced, leading to an enhanced excitability that can provoke the development of ventricular tachycardia or fibrillation (12). In the present study, we found that puFAs could suppress the excitability by increasing the threshold potential (more positive), the resting membrane potential (more negative), and the cycle length

Table 1. Effect of EPA on the properties of action potential in neonatal rat cardiac myocytes

	RMP, mV	APT, mV	APA, mV	V_{max}, V/s	APD, ms
Control	−52	−52	101	57	204
EPA	−57	−43	99	54	163
Δ	5 ± 1*	9 ± 3*	3 ± 1†	3 ± 2†	45 ± 12‡

For each data column, $n = 8$. The parameters for EPA were measured 5 min after 10 μM EPA addition. RMP, resting-membrane potential; APT, action-potential threshold; APA, action-potential amplitude; V_{max}, maximum speed of phase 0 depolarization; APD, action-potential duration at 75% repolarization. Δ, Absolute values of the difference between control and EPA values.

*$P < 0.05$.

†P value not statistically significant.

‡$P < 0.01$.

(refractory period), all of which oppose the adverse effects induced by ischemia. puFA, therefore, may serve to reduce myocardial substrate vulnerability to arrhythmogenic stimuli by enhancing myocardial stability. Thus, the relevance of the present findings relate to the protective effects of puFA on ischemia-induced arrhythmias.

There has been much concern regarding the potential deleterious effects of free fatty acids that accumulate during cardiac ischemia (for review, see refs. 13–15). The present study indicates the need to be quite specific regarding the effects of free fatty acids on cardiac function. The *cis*-puFAs studied here, in contrast to saturated and monounsaturated fatty acids, have acute, potentially protective antiarrhythmic actions. Because the ω3 puFA have been persistently antiarrhythmic, whereas arachidonic acid (ω6) can produce tachyarrhythmias via its oxidized metabolites (9), it will probably prove prudent to restrict potential antiarrhythmic uses to the ω3 class of puFA. It is evident that there exists an important basic level of control of cardiac function dependent upon choices of fatty acids in the diet that has been, with few exception (16, 17), largely overlooked.

The excitability/automaticity of cardiac cells depends on several membrane currents. The ionic currents responsible for the observed effects have not been identified in this study. Normally, the ionic currents that contribute to the automaticity include an inward current, I_f; an inward Ca^{2+} current, I_{Ca}; and an outward K^+ current, I_k (11). The two inward currents, I_f and I_{Ca}, are responsible for progressive phase 4 depolarization, whereas the outward K^+ current opposes the depolarizing effects of the inward currents. Perhaps the EPA-induced increase in the resting membrane potential (Table 1) and slow rate of phase 4 depolarization (Fig. 4) may be due to an inhibition of I_f and I_{Ca} and/or an enhancement of I_k. The more positive action-potential threshold induced by puFA (Fig. 2 and Table 1) indicates an effect of the fatty acids on the gating of the fast Na^+ channel. The EPA-induced shortening of action-potential duration (Fig. 2 and Table 1) may result from inhibition of voltage-dependent L-type Ca^{2+} channels and/or augmentation of K^+ channels. Abbreviation of the action-potential duration generally shortens refractoriness. However, in the present study, the cycle length, which reflects the refractory period, was significantly prolonged despite a shortened action-potential duration (Fig. 3). This result may be due to a delay in recovery of fast Na^+ channels as a result of an action of EPA together with the prolonged phase 4 depolarization, as mentioned. Thus, it seems that puFAs modulate the myocardial excitability by affecting several kinds of ion channels. We have tested the effects on the action potential of various ion-channel blockers, including nitrendipine (a Ca^{2+}-channel blocker), tetrodotoxin (a Na^+-channel blocker), 4-aminopyridine (a K^+-channel blocker), and diphenylamine 2-carboxylate (a Cl^--channel blocker). Our preliminary results (data not shown) indicate that none of these blockers could singly mimic the entire effects of EPA, suggesting that EPA probably affects more than one ion channel.

Recent reports have shown that fatty acids have a wide variety of effects on membrane currents in excitable tissues—including cardiac muscle (18–22), neurons (23–26), smooth muscle (27, 28), and skeletal muscle (29). In heart cells, Pepe *et al.* (18) found that 5 μM DHA inhibited the effect of dihydropyridine agonists and antagonists on Ca^{2+}-channel currents but reported no significant effect alone on the channel in adult rat cardiac myocytes; Huang *et al.* (19) reported that long-chain unsaturated and saturated fatty acids (3–30 μM) increased voltage-dependent Ca^{2+} currents in guinea pig ventricular myocytes; Kim and Clapham (20) found that arachidonic acid and other unsaturated, but not saturated, fatty acids (10–50 μM) activated outwardly rectifying K^+ channels in rat ventricular cells, whereas Honore *et al.* (22) recently showed that arachidonic acid and DHA inhibited the major cardiac delayed-rectifier K^+ channels in mouse and rat cardiomyocytes. However, the effect of fatty acids on sodium channel in heart cells has not been reported. In muscle and neural cells, outward rectifying K^+ channels are activated (27, 28), whereas Na^+ channels are inhibited (25, 29) by certain fatty acids. Thus, although the effects of increased extracellular free fatty acids may vary depending upon the type and concentration of fatty acid, experimental conditions, and cell type, certain fatty acids probably serve to reduce membrane electrical excitability in excitable cells by activating K^+ channels and inhibiting Na^+ channels. However, other possibilities, such as effects on Ca^{2+} or Cl^- channels, are not excluded. Obviously, further study is warranted to identify the ion channels responsible for the observed effects of the fatty acids and to characterize their effects on activities of the individual ion channel affected.

The present observations that (*i*) the electrophysiologic effects of EPA occurred so quickly and could be reversed by bovine serum albumin and (*ii*) nonmetabolizable ETYA also had similar effects suggest that the effects of puFAs do not rely on their metabolites or incorporation into phospholipids, consistent with their effects on cardiomyocyte contraction rate and arrhythmias (9, 10). puFAs appear to directly modulate some ion channels by an interaction with the ion-channel protein itself or by acting at some lipid sites near the channels after partition into lipid bilayer. Further study in this regard is needed.

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1. Burr, M. L., Fehily, A. M., Gilbert, J. F., Rogers, S., Holliday, R. M., Sweetnam, P. W., Elwood, P. C. & Deadman, N. M. (1989) *Lancet* **334,** 757–761.
2. de Logeril, M., Renaud, S., Mamelle, N., Salen, P., Martin, J.-L., Monjaud, I., Guidollet, J., Touboul, P. & Delaye, J. (1994) *Lancet* **343,** 1454–1459.
3. McLennan, P. L., Abeywardena, M. Y. & Charnock, J. S. (1988) *Am. Heart J.* **16,** 709–717.
4. McLennan, P. L. (1993) *Am. J. Clin. Nutr.* **57,** 207–212.
5. McLennan, P. L., Bridle, T. M., Abeywardena, M. Y. & Charnock, J. S. (1992) *Am. Heart J.* **123,** 1555–1561.
6. Billman, G. E., Hallaq, H. & Leaf, A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 4427–4430.
7. Hallaq, H., Sellmayer, A., Smith, T. W. & Leaf, A. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 7834–7838.
8. Hallaq, H., Smith, T. W. & Leaf, A. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1760–1764.
9. Kang, J. X. & Leaf, A. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 9886–9890.
10. Kang, J. X. & Leaf, A. (1995) *Biochem. Biophys. Res. Commun.*, in press.
11. Berne, R. M. & Levy, M. N. (1993) in *Physiology*, eds. Berne, R. M. & Levy, M. N. (Mosby Year Book, Boston), 3rd Ed., pp. 364–396.
12. Victor, E. & Zipes, D. (1977) *Am. J. Physiol.* **233,** H329–H345.
13. Katz, A. M. & Messineo, F. C. (1981) *Circ. Res.* **48,** 1–16.
14. Corr, P. B., Gross, R. W. & Sobel, B. E. (1984) *Circ. Res.* **55,** 135–145.
15. Van Der Vusse, G. J., Glatz, J. F. C., Stam, H. C. G. & Reneman, R. S. (1992) *Physiol. Rev.* **72,** 881–940.
16. Gudbjarnason, S. (1989) *J. Intern. Med.* **225,** Suppl. 1, 117–128.
17. Charnock, J. S., Sundram, K., Abeywardena, M. Y., McLennan, P. L. & Tan, D. T. (1991) *Am. J. Clin. Nutr.* **53,** 1047S–1049S.
18. Pepe, S., Bogdanov, K., Hallaq, H., Spurgeon, H., Leaf, A. & Lakatta, E. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 8832–8836.
19. Huang, J. M. C., Xian, H. & Bacaner, M. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6452–6456.

20. Kim, D. & Clapham, D. (1989) *Science* **244,** 1174–1179.
21. Kim, D. & Duff, R. A. (1990) *Circ. Res.* **67,** 1040–1046.
22. Honore, E., Barhanim, J., Attali, B., Lesage, F. & Lazdunski, M. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 1937–1944.
23. Rouzaire-Dubois, B., Gerard, V. & Dubois, J. M. (1991) *Pflügers Arch.* **419,** 467–471.
24. Mill, B., Sarantis, M., Traynelis, S. F. & Attwell, D. (1992) *Nature (London)* **355,** 722–725.
25. Linden, D. J. & Routtenberg, A. (1989) *J. Physiol. (London)* **419,** 95–119.
26. Takenaka, T., Horie, H., Hori, H. & Kawakami, T. (1988) *J. Membr. Biol.* **106,** 141–147.
27. Ordway, R. W., Walsh, J. V. & Singer, J. J. (1989) *Science* **244,** 1176–1179.
28. Kirber, M. T., Ordway, R. W., Clapp, L. H., Walsh, J. V. & Singer, J. J. (1992) *FEBS Lett.* **297,** 24–28.
29. Wieland, S. J., Fletcher, J. E., Gong, O. H. & Rosenberg, H. (1991) in *Mechanisms of Anesthetic Action in Skeletal, Cardiac and Smooth Muscle*, eds. Blanck, T. J. J. & Wheeler, D. M. (Plenum, New York), pp. 9–19.