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ARACHIDONIC ACID-INDUCED Ca^{2+} RELEASE FROM ISOLATED SARCOPLASMIC RETICULUM

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Abstract—Arachidonic acid has been shown to release Ca^{2+} from isolated skeletal and cardiac sarcoplasmic reticulum (SR) vesicles. The release took place nearly equally well from all fractions of the SR and was only partially inhibited by ruthenium red, suggesting that some other pathway is involved in addition to the SR Ca^{2+} release channel. Arachidonic acid increased SR Ca^{2+} efflux even in the presence of several different SR Ca^{2+} pump inhibitors. It also had considerably less effect on uptake measured in the presence of oxalate and did not appear to inhibit Ca^{2+}-dependent ATPase activity. Thus, the SR Ca^{2+} pump also appears to be minimally perturbed by arachidonic acid. Arachidonyl CoA was more effective at releasing Ca^{2+} than the parent compound. Arachidonic acid effects were not inhibited by lipoxygenase or cyclooxygenase inhibitors, suggesting that no eicosanoids are involved in the effects under study here. Flunarizine, cinnarizine and propyl-methylenedioxyindene inhibited the Ca^{2+} release induced by arachidonic acid. The effects of arachidonic acid appear to depend on the ratio of arachidonic acid to membrane vesicles.

Fatty acids are known to accumulate in a variety of pathological states, including cardiac ischemia [1–3] and dystrophic chicken skeletal muscle [4]. Fatty acids have also been proposed to be involved in the pathogenesis of malignant hyperthermia in skeletal muscle [5, 6]. They have been shown to impair cardiac muscle function in normal and ischemic hearts [7, 8] and to depress contractile function in skeletal and smooth muscles as well [9]. Arachidonic acid has been shown to mobilize Ca^{2+} from intracellular stores in a variety of different cell types [10–12] and to liberate Ca^{2+} from internal membranes isolated from several tissue types [13, 14].

Arachidonic acid and other fatty acids have long been known to interfere with Ca^{2+} accumulation by isolated sarcoplasmic reticulum (SR)† [15–18]. These earlier studies made few attempts to determine whether the reduced net Ca^{2+} uptake rates measured were due primarily to inhibition of the SR Ca^{2+} pump as opposed to increased efflux through SR Ca^{2+} release channels or some other pathway. The present study will elucidate this issue.

METHODS

Terminal cisternae and light SR subfractions were prepared according to Saito *et al.* [19]. Canine cardiac microsomes were prepared according to Harigaya and Schwartz [20] with minor modifications (omission of a salt wash). While these microsomes were not free of mitochondrial contamination and mitochondria have been reported to release Ca^{2+} in the presence of fatty acids [21], the releases reported here also took place when 5 mM azide was added to the assay medium prior to Ca^{2+} loading of the microsomes. Such concentrations of azide selectively inhibit mitochondrial Ca^{2+} uptake [20]. Since arachidonic acid induced release of the majority of Ca^{2+} accumulated by these microsomal membranes, and was also capable of releasing Ca^{2+} from highly purified skeletal SR subfractions, we consider mitochondrial contribution to any of the effects reported here to be minimal.

Ca^{2+} release was studied spectrophotometrically by modifications of Palade [22], with phosphate substituted for pyrophosphate as a precipitating anion. Accordingly, canine cardiac and rabbit skeletal microsomes as well as purified rabbit skeletal SR subfractions (0.15 to 0.50 mg protein/mL) were loaded at 36° with various amounts of $CaCl_2$ in the presence of 90 mM KCl, 18 mM potassium-4-morpholinepropanesulfonic acid (K-MOPS), 5 mM potassium-phosphate, 1 mM MgATP, 5 mM disodium phosphocreatine, 20 μg/mL creatine phosphokinase, and 0.25 mM antipyrylazo III, pH 7.0. Following the completion of Ca^{2+} uptake, arachidonic acid was added from a concentrated stock to the 1 mL solution. Ca^{2+} movements were monitored by measurement of the absorbance at 710 nm and subtraction of the absorbance at 790 nm at either 1- or 2-sec intervals in a Hewlett–Packard 8451A diode array spectrophotometer. The dye absorbance increases at 710 nm but not at 790 nm when it binds Ca^{2+}; the subtraction of A_{790} serves to correct for changes in light scattering due to possible vesicle shrinking or swelling, which would contribute at both wavelengths.

Ca^{2+} uptake by canine cardiac and rabbit skeletal microsomes was also studied spectrophotometrically using conditions identical to those for the Ca^{2+} release studies except for the use of 5 mM oxalate instead of phosphate in order to minimize Ca^{2+}

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† Abbreviations: AA, arachidonic acid; A CoA, arachidonyl CoA; MOPS, 4-morpholinepropanesulfonic acid; RR, ruthenium red; and SR, sarcoplasmic reticulum.

efflux. Ca^{2+}-ATPase was monitored in the same solution under the same conditions with microsomes rendered leaky using the Ca^{2+} ionophore A23187 (2 μM) so as to negate indirect effects of increased Ca^{2+} efflux on the ATPase determinations. Ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA) was only added for basal determinations, but the presence of A23187 resulted in a time-invariant [Ca^{2+}] during determinations performed in the absence of EGTA. Reactions were terminated using 10% trichloroacetic acid, and phosphate was determined according to Ottolenghi [23]. ATPase activity was determined in the presence of various concentrations of $CaCl_2$. Basal ATPase levels were determined in the presence of 1 mM EGTA with no added Ca^{2+}.

Determinations of unidirectional ^{45}Ca efflux were performed using the release assay described above, with the following modifications. First, ^{45}Ca was loaded into the sample instead of unlabeled calcium. Second, 150-μL aliquots of the medium were removed from the cuvette, filtered through Millipore 0.45 μm nitrocellulose filters, and washed twice with 3 mL of chilled 100 mM KCl, 5 mM potassium-phosphate, 20 mM K-MOPS, 3 μM ruthenium red and 1 mM EGTA, pH 7.0. The protocol involved loading the sample with three 6.25-nmol aliquots of ^{45}Ca and filtering one aliquot of the medium after spectrophotometrically monitored uptake had been completed. Third, 1 mM EGTA with or without 50 μM arachidonic acid was added to the cuvette, which represented zero time. Subsequently, further aliquots were filtered and washed after 15, 30, 60 and 120 sec.

Statistical significance of the differences between the means of two populations was determined by paired or unpaired *t*-tests, as specified, with significance determined at the 95% confidence level.

Arachidonic acid was obtained from Sigma and was prepared as a concentrated stock solution in ethanol, in which it was frozen in small aliquots in liquid nitrogen. No more than 10 μL ethanol (1%) was added to SR in any experiment. At these concentrations, ethanol by itself caused no Ca^{2+} release from any of the SR samples under these experimental conditions. Cyclopiazonic acid and thapsigargin stock solutions were prepared in dimethyl sulfoxide. 2,5-Di-t-butylhydroquinone was obtained from Aldrich and carbocyclic thromboxane A_2 (CTA_2) from Calbiochem. Other reagents were obtained from Sigma or Fisher Scientific in the highest purity available. Propyl-methylenedioxyindene was a gift from Dr. R. Rahwan, Ohio State University.

RESULTS

If either skeletal or cardiac microsomal vesicles are permitted to take up Ca^{2+} in the presence of phosphate ion and then are exposed to relatively high concentrations of arachidonic acid (>10 μM), they will release a large portion of their accumulated Ca^{2+} (Fig. 1). Even with 50 μM arachidonic acid, the release of Ca^{2+} from cardiac microsomes took place more slowly (0.28 μmol · mg^{-1} · min^{-1}) than that induced by 10 mM caffeine (0.59 μmol · mg^{-1} · min^{-1}) under comparable conditions (not shown). Arachidonic acid was able to release a smaller amount of Ca^{2+} from samples preloaded only with their endogenous Ca^{2+} (not shown, equivalent to one of the additions in Fig. 1A). Far greater preloading (nine such additions) was required before a further Ca^{2+} addition of the same size elicited a Ca^{2+}-induced Ca^{2+} release under these conditions (not shown). With the degree of preloading shown in Fig. 1A, more than 50 nmol of $CaCl_2$ must be added to elicit Ca^{2+}-induced Ca^{2+} release (not shown).

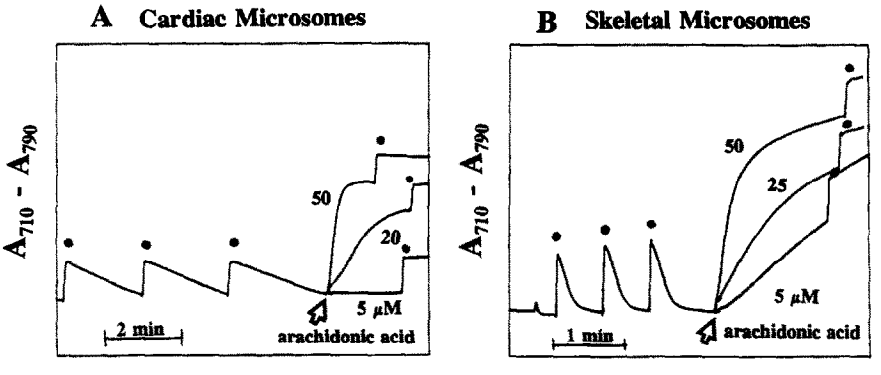

Fig. 1. Arachidonic acid-induced Ca^{2+} release from muscle microsomal membranes. (A) Canine cardiac microsomes (0.15 mg protein) were administered three 6.25-nmol aliquots of $CaCl_2$ (the three small rapid upward deflections marked by dots at the start of each trace) at 36° in 1.0 mL of phosphate-containing assay medium. The traces represent Ca^{2+} concentration changes monitored by changes in antipyrylazo III absorbance at 710 nm corrected for light scattering changes by subtraction of absorbance at the Ca^{2+}-independent wavelength 790 nm. The upward deflections are followed by slow downward movements in the trace which represent Ca^{2+} uptake by the microsomal vesicles. Following the uptake of the third aliquot, arachidonic acid was added at concentrations of 5, 20, and 50 μM, as indicated. The final upward deflection at the end of each trace represents another 6.25 nmol $CaCl_2$ addition made for calibration purposes in the presence of the arachidonic acid. (B) Rabbit skeletal microsomes (0.15 mg) were administered three 12.5-nmol $CaCl_2$ additions as described above and subsequently exposed to 5, 25, and 50 μM arachidonic acid, as indicated. Note the difference in time scales.

Arachidonic acid-induced Ca^{2+} release took place at similar rates with purified skeletal muscle SR subfractions derived either from terminal cisternae enriched in Ca^{2+} release channels or light SR derived from longitudinal SR lacking such channels (Fig. 2). Releases induced by arachidonic acid from different cardiac and skeletal SR samples were variably affected by ruthenium red, a release channel blocker, but in all cases significant release rates were observed even in the presence of ruthenium red (Fig. 2C). These results would suggest that the SR Ca^{2+} release channel is not the only pathway activated by arachidonic acid, and that some other constituent of the SR membrane, one present throughout the SR, is affected.

The relatively slow rate of Ca^{2+} release caused by arachidonic acid could be accounted for if it inhibited the SR Ca^{2+} pump, unmasking a relatively high leak of Ca^{2+} across the membrane. To test this possibility, we performed determinations with oxalate substituted for phosphate in our assay medium. Both oxalate and phosphate promote SR Ca^{2+} uptake by precipitating Ca^{2+} inside the SR lumen, preventing accumulated Ca^{2+} from inhibiting the pump. As seen in Fig. 3B, arachidonic acid slowed but did not prevent Ca^{2+} uptake in the presence of oxalate, and it did not cause a release of Ca^{2+} under these conditions. These results were found both for skeletal and cardiac microsomes treated in precisely the same fashion as in Fig. 1 except with oxalate substituted for phosphate. Under these same conditions, the ionophore A23187 rapidly released Ca^{2+} from the vesicles (Fig. 3B), indicating that the physical state of calcium-oxalate precipitate in the vesicles was not responsible for the failure of arachidonic acid to release Ca^{2+}.

Oxalate is known to reduce the intralumenal free [Ca^{2+}] more than phosphate [24]. Accordingly, it could have reduced the inhibitory effects of arachidonic acid in one of two ways, either by reducing the magnitude of an arachidonic acid-induced Ca^{2+} leak out of the SR or, alternatively,

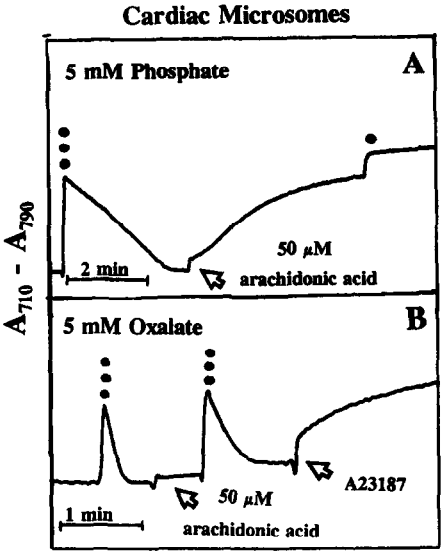

Fig. 3. Lesser effectiveness of arachidonic acid in the presence of oxalate. (A) Canine cardiac microsomes (0.15 mg) were exposed to an 18.75-nmol addition of $CaCl_2$ and then subsequently to 50 μM arachidonic acid where indicated, resulting in a small, slow release of Ca^{2+}. Subsequent addition of another 6.25 nmol $CaCl_2$ was unable to elicit further Ca^{2+} uptake under these conditions. (B) Canine cardiac microsomes (0.45 mg) were exposed to a single addition of 18.75 nmol $CaCl_2$ under conditions identical to those described for Fig. 1, except that 5 mM oxalate was substituted for 5 mM phosphate. Addition of 50 μM arachidonic acid produced no release and only slightly slowed the uptake of a subsequent addition of 18.75 nmol $CaCl_2$. Subsequent addition of 2 μM A23187 did produce Ca^{2+} release under these conditions.

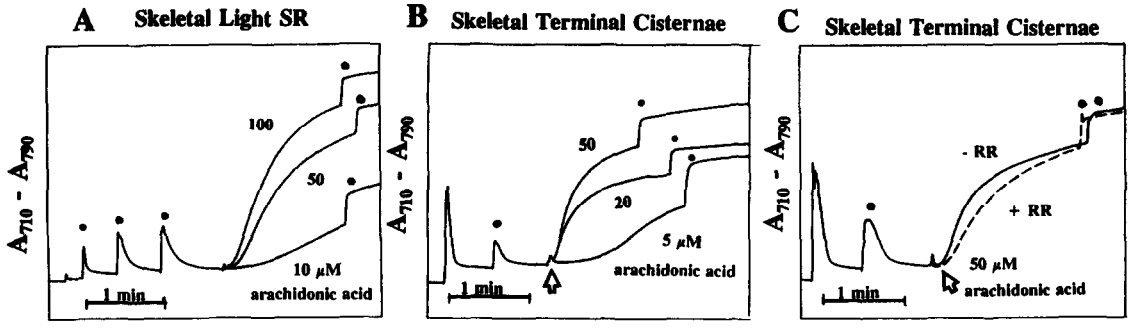

Fig. 2. Arachidonic acid-induced Ca^{2+} release from purified subfractions of rabbit skeletal muscle sarcoplasmic reticulum. (A) Purified light SR (0.45 mg) was loaded with three 12.5-nmol additions of $CaCl_2$ at 36° as described for Fig. 1 and subsequently exposed to 10, 50, and 100 μM arachidonic acid as indicated. (B) Purified terminal cisternae (0.45 mg) were loaded with an identical total amount of Ca^{2+} as the light SR in the previous panel (6.25 nmol plus 31 nmol endogenous Ca^{2+} in the sample) and subsequently exposed to 5, 20, and 50 μM arachidonic acid, as indicated. (C) Purified terminal cisternae were treated in an identical fashion with 50 μM arachidonic acid (solid trace), or by an addition of 1 μM ruthenium red (RR) made just prior to the arachidonic acid addition (dashed trace). Final upward deflections in all cases represent Ca^{2+} recalibrations in the presence of the arachidonic acid.

by reducing the inhibitory influence of free internalized Ca^{2+} on the pump. To distinguish between these possibilities, we assayed ATP hydrolysis by leaky SR vesicles in the presence of different Ca^{2+} concentrations. As seen in Fig. 4, arachidonic acid had no profound influence on the rate of ATP hydrolysis at any Ca^{2+} concentration tested. This result suggested that arachidonic acid was not sensitizing the pump to the deleterious effects of internalized Ca^{2+} but instead was rendering the membrane more leaky to Ca^{2+}.

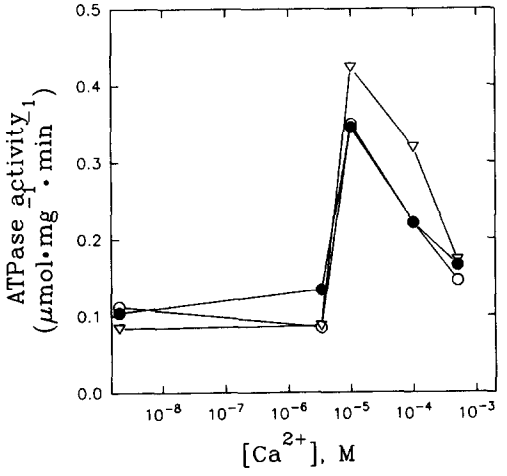

Fig. 4. Minimal effects of arachidonic acid on Ca^{2+}-stimulated ATPase activity. ATPase activity of leaky canine cardiac microsomes (0.2 mg/mL) was measured in the absence of precipitating anions as described in Methods in the presence of various concentrations of Ca^{2+}. The rate of ATP hydrolysis is plotted as a function of Ca^{2+} concentration in the presence of 2.5 μM (●) and 200 μM arachidonic acid (▽) and in its absence (○). The experiment was performed once at the two lower Ca^{2+} concentrations and in duplicate with the same sample at the three higher $[Ca^{2+}]$. Using a paired *t*-test, no statistically significant difference could be detected between any of the data points at 10, 100 or 500 μM Ca^{2+}.

To test for this possibility, cardiac microsomal vesicles were loaded with ^{45}Ca under the same conditions utilized in Fig. 1. After Ca^{2+} loading had been completed, 1 mM EGTA was added to the cuvette solution together with sufficient unlabeled Ca^{2+} to maintain the absorbance baseline (free $[Ca^{2+}]$) at the same value, and aliquots were removed from the cuvette at timed intervals. These aliquots were immediately filtered through Millipore filters and the ^{45}Ca retained on the filters was determined by liquid scintillation spectrometry. As seen in Fig. 5, the rate of Ca^{2+} efflux under these conditions was very slow, far too slow by itself to account for the releases seen in Fig. 1 if the only effect of the arachidonic acid had been to inhibit pump-mediated unidirectional Ca^{2+} influx. The addition of 50 μM arachidonic acid promoted a large increase in the measured efflux rate, confirming that arachidonic acid made the membrane significantly more permeable to Ca^{2+}. This increased efflux was also observed when 3 μM ruthenium red was added prior to the addition of arachidonic acid.

The failure of ruthenium red to inhibit the increased ^{45}Ca efflux induced by arachidonic acid, together with the extensive releases elicited from SR subfractions with low SR Ca^{2+} release channel content, suggest that the efflux is mediated by some constituent distributed throughout the SR. The most obvious candidate for such a role is the SR Ca^{2+} pump. While the Ca^{2+}-ATPase measurements clearly indicated that the vast majority of pump molecules were still hydrolyzing ATP in the presence of arachidonic acid, a few pump molecules could still have been modified into a large leak pathway. We tested for such a possibility by blocking the pump, and presumably freezing it in certain different conformations, with several different inhibitors. Ruthenium red was present to prevent possible release channel activation by certain of the inhibitors (e.g. [25]). Neither 200 μM quercetin [26], 100 μM 2,5-di-t-butylhydroquinone [27], 50 μM cyclopiazonic acid [28], nor 1 μM thapsigargin [29] added 0.5 to 5 min prior to arachidonic acid addition was able to substantially slow the release of Ca^{2+} elicited by the arachidonic acid (not shown).

We have tested numerous other compounds for their ability to suppress arachidonic acid-induced Ca^{2+} release. The thromboxane receptor antagonist CTA_2 (20 μM) [30] had no effect, nor did metapyrone (500 μM) or SKF 525a (100 μM), two cytochrome P450 monooxygenase inhibitors [31]. Neither 200 μM acetylsalicylate, 100 μM indomethacin [32], 70 μM nordihydroguaiaretic acid [32], nor 10 μM mepacrine prevented the release. Combinations of these arachidonic acid metabolism inhibitors (50 μM indomethacin + 30 μM nordihydroguaiaretic acid + 100 μM metapyrone) were similarly ineffective, as were the vast majority of more than two hundred substances tested (not shown). Only flunarizine (not shown), cinnarizine and propyl-methylenedioxy-indene [33] appeared to be effective inhibitors (Fig. 6). These substances were equally effective at inhibiting arachidonic acid-induced Ca^{2+} release from skeletal muscle SR membranes (not shown).

Since prolonged exposure to inhibitors of fatty acyl synthetase (malonyl CoA or acetyl CoA under defined conditions [34]) reduced the ability of the SR to take up Ca^{2+}, we were unable to determine whether fatty acyl esters were involved in the arachidonic acid effects reported here. However, arachidonyl CoA was more potent than its parent compound at inducing Ca^{2+} release (Fig. 7).

The concentrations of arachidonic acid employed in the study to this point were higher than expected to occur under even extreme conditions of ischemia. Accordingly, we investigated the impairment of net SR Ca^{2+} uptake in the presence of lower concentrations of arachidonic acid. As seen in Fig. 8, concentrations of arachidonic acid too low to induce SR Ca^{2+} release were nevertheless sufficient to reduce SR Ca^{2+} uptake markedly. These determinations were also performed in the presence

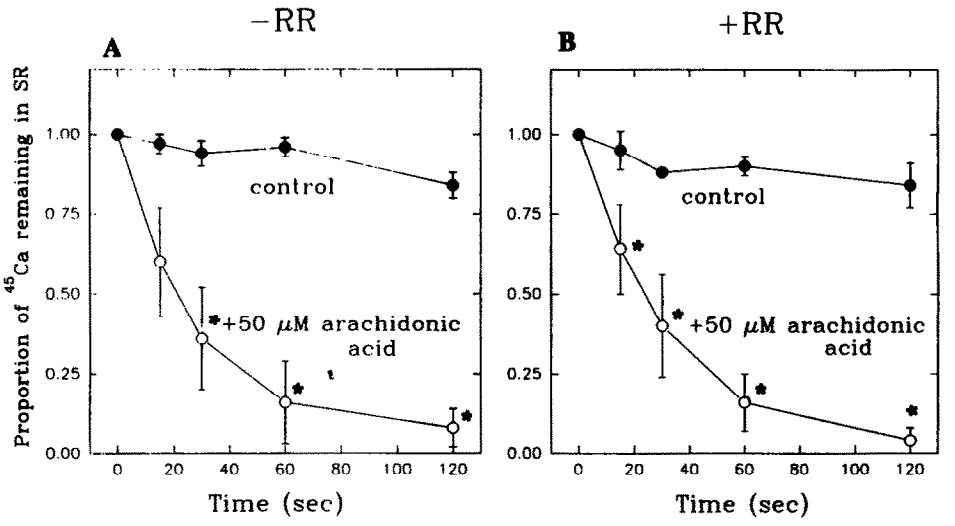

Fig. 5. Stimulation of ^{45}Ca efflux from canine cardiac microsomes by arachidonic acid. Following uptake of three 6.25-nmol aliquots of ^{45}Ca by cardiac microsomes (0.15 mg in 1 mL), 1 mM EGTA was added to the cuvette with or without 50 μM arachidonic acid and aliquots were filtered at the times indicated as outlined in Methods. In panel B, 3 μM ruthenium red (RR) was present throughout the assay. The experiments were repeated in triplicate on the same sample with error bars representing standard deviations. Asterisks denote a statistically significant difference compared to the control time point at the $P < 0.05$ level, using a paired *t*-test.

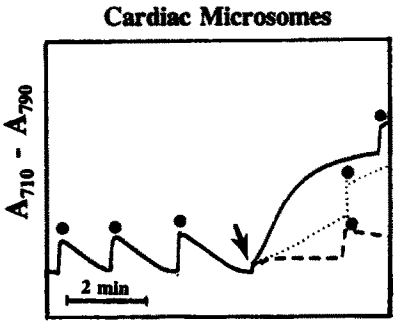

Fig. 6. Inhibition of arachidonic acid-induced Ca^{2+} release by certain substances. Cardiac microsomes (0.2 mg) were loaded with three 6.25-nmol aliquots of $CaCl_2$ and then exposed to 50 μM arachidonic acid at the arrow, either alone (solid trace) or shortly after the addition of 1 μM propyl-methylenedioxyindene (dotted trace) or 100 μM cinnarizine (dashed trace).

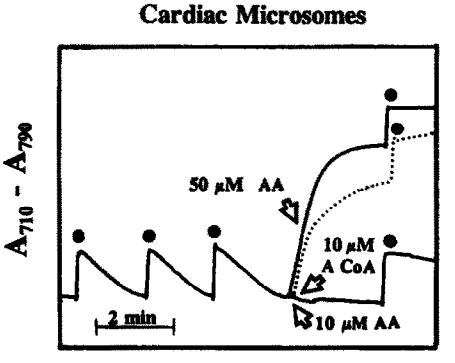

Fig. 7. Relative potencies of arachidonyl CoA and arachidonic acid at inducing Ca^{2+} release. Cardiac microsomes (0.2 mg protein) were suspended in the same solution described for Fig. 1. Three 6.25-nmol $CaCl_2$ additions were then administered, followed by either 10 or 50 μM arachidonic acid (solid traces, AA) or 10 μM arachidonyl CoA (dotted trace, A CoA).

of 3 μM ruthenium red to block any SR Ca^{2+} release channels that happened to be open under the conditions of the release assay.

If such low concentrations of arachidonic acid were able to impair SR function significantly, then they might contribute directly to the untoward effects of ischemia on myocardial tissue. However, arachidonic acid is a very lipophilic substance, and prior reports [5, 17] had quantitated its potency in terms of amount of arachidonic acid relative to the amount of membrane protein present. Accordingly, we determined the effects of arachidonic acid on Ca^{2+} release at two different concentrations of SR. As seen in Fig. 9, 20 μM arachidonic acid only caused release in the experiment involving the smaller amount of sample, whether preloading was accomplished with one larger Ca^{2+} addition (A) or five smaller ones each one-fifth the amount (not shown). We also assessed effects on Ca^{2+} uptake with the assay system described in Fig. 8 using two different amounts of cardiac microsomal vesicles in the cuvette. By subtracting the rate of net uptake in the absence of ruthenium red from the faster rate

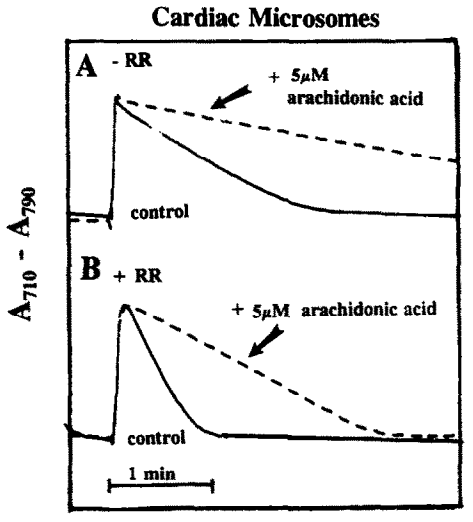

Fig. 8. Reduction of net Ca^{2+} uptake by arachidonic acid in the presence or absence of ruthenium red (RR). In the upper pair of traces, cardiac microsomes (0.2 mg protein) were suspended in the same solution described for Fig. 1. An addition of 6.25 nmol $CaCl_2$ was then made and the rate of net Ca^{2+} uptake measured in the absence (solid trace) or presence (dashed trace) of 5 μM arachidonic acid. In the lower pair of traces, the same procedure was followed in the presence of 3 μM RR in the absence (solid trace) or presence (dashed trace) of 5 μM arachidonic acid. Arachidonic acid and RR additions were made shortly before the commencement of the traces shown.

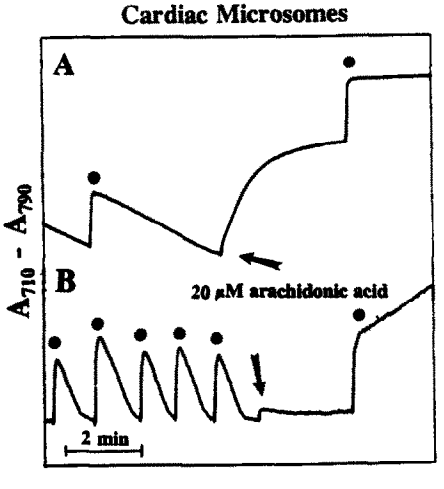

Fig. 9. Dependence of arachidonic acid-induced Ca^{2+} release on the amount of membranes present. (A) Canine cardiac microsomes (0.125 mg) were preloaded with 6.25 nmol $CaCl_2$ and then challenged with 20 μM arachidonic acid. (B) The experiment was repeated with 0.625 mg canine cardiac microsomes loaded with correspondingly more $CaCl_2$ (5 × 6.25 nmol). Note that uptake was slower in the presence of less sample (A), and that release of Ca^{2+} occurred only in this case, as well.

occurring in its presence, we could also determine the rate of ruthenium red-sensitive, release channel-mediated efflux. As seen by the results presented in Table 1, if more SR was present in the cuvette, a correspondingly higher concentration of arachidonic acid was required to produce its untoward effects on either net SR Ca^{2+} uptake or on the release channel-mediated efflux. Similarly, if only one-third the amount of protein employed in Fig. 3 was used, 50 μM arachidonic acid could produce effects on net uptake and even generate some slight Ca^{2+} release in the presence of oxalate (not shown). These results suggest that the critical determinant of arachidonic acid effects is not its concentration in the aqueous environment but rather its concentration in the membrane phase.

DISCUSSION

The results obtained here clearly demonstrate that arachidonic acid is able to impair net Ca^{2+} uptake by the SR. While some activation of release channels may occur under some circumstances, these effects are likely to involve a secondary Ca^{2+}-induced Ca^{2+} release triggered by an elevation of Ca^{2+} produced through some other pathway. This other pathway is apparently distributed throughout the SR, since equivalent release of Ca^{2+} was elicited from skeletal SR subfractions obtained from different regions of the SR. It is unlikely to involve the SR Ca^{2+}-ATPase, since there was much less effect on oxalate-supported Ca^{2+} uptake and negligible reduction of ATPase activity by arachidonic acid. In addition, several different pump inhibitors failed to affect the release of Ca^{2+} elicited by arachidonic acid. We do not believe a detergent-like solubilization of the membrane is involved, since arachidonic acid did not release Ca^{2+} from oxalate-loaded vesicles, even under conditions where the ionophore A23187 did. Most likely some non-pump, non-release channel protein component of the membrane was affected, but we have not been able to ascertain which component that might be. A carrier-type mechanism has been hypothesized to account for arachidonic acid-induced Ca^{2+} efflux from platelet microsomes [35]. An alternative possibility is that arachidonic acid merely renders the lipid portion of the bilayer more leaky to Ca^{2+}.

It is highly unlikely that prostaglandin, eicosanoid or leukotriene metabolites of arachidonic acid are involved in the effects described here. First, the release of Ca^{2+} produced by arachidonic acid was virtually insensitive to indomethacin or acetylsalicylate (inhibitors of the cyclooxygenase pathway [cf. Ref. 32]), to nordihydroguaiaretic acid (not shown), inhibitor of the lipoxygenase pathway [cf. Ref. 32], to metapyrone or SKF 525A (inhibitors of cytochrome P450 monooxygenease [31]), or to CTA_2 (a thromboxane receptor antagonist [30]). Second, numerous other fatty acids which are not metabolized into eicosanoids are similarly able to produce Ca^{2+} release from the SR (Dettbarn C and Palade P, unpublished results; [17]). These include saturated fatty acids such as palmitic acid, which yields a dose–response relationship similar to that of arachidonic acid, and arachidic acid, which is less

Table 1. Effects of arachidonic acid (AA) on sarcoplasmic reticulum Ca^{2+} uptake and release as a function of the amount of membranes present

	SR Ca^{2+} uptake ($nmol \cdot mg^{-1} \cdot min^{-1}$)		RR-sensitive efflux ($nmol \cdot mg^{-1} \cdot min^{-1}$)
	−RR	+RR	
0.2 mg Membrane protein			
Control	18.3 ± 3.5 (8)	45.5 ± 5.8 (3)	27.2
+20 μM AA	0.7 ± 7.9 (5)	4.1 ± 8.6 (4)	3.4
1.0 mg Membrane protein			
Control	17.0 ± 4.0 (7)	42.1 ± 0.8* (2)	25.1
+20 μM AA	10.6 ± 3.9 (7)	31.3 ± 9.4 (3)	20.7

Values are means ± SD, except where indicated by an asterisk (*) in which case the value represents the mean ± range. Numbers in parentheses refer to the number of observations, which were carried out with several different microsomal samples. Abbreviations: RR, ruthenium red; and AA, arachidonic acid. Using an unpaired Student's *t*-test, there was no statistically significant difference between the two −RR controls or the two +RR controls. There was a statistically significant difference between the two −RR determinations in the presence of AA and between the two +RR determinations in the presence of AA ($P < 0.05$).

effective (not shown). Such effects are more marked than those reported for PGI_2 [36]. However, other metabolites of arachidonic acid, such as fatty acyl CoA derivatives [37], may be involved in the release of Ca^{2+}, particularly since these compounds appear more potent than their parent fatty acids.

The ability of flunarizine and cinnarizine to inhibit the Ca^{2+} release produced by arachidonic acid may contribute to the protective action of these Ca^{2+} entry blocking agents against ventricular fibrillation [38], cerebrovascular disorders [39] and neuronal cell death [40]. Indeed, arachidonic acid at similar concentrations was able to release Ca^{2+} accumulated by isolated brain and uterine smooth muscle membranes (Dettbarn C and Palade P, unpublished observations). Propyl-methylenedioxyindene has been reported to produce a negative inotropic effect on heart muscle [33].

The documented Ca^{2+}-releasing properties reported here could lead to a net loss of Ca^{2+} from the SR, particularly since arachidonic acid has also been reported to stimulate the cardiac sarcolemmal Na^+/Ca^{2+} exchanger [41]. This could contribute to the negative inotropic effects associated with administration of arachidonic acid to heart tissue [7, 8], particularly since ventricular myocyte calcium currents are reportedly increased by arachidonic acid [42]. If release of calcium from SR instead led to elevations of free myoplasmic $[Ca^{2+}]$, this might contribute to decreases in gap junction coupling produced by arachidonic acid in heart cells [43].

The observation that both inhibitory effects of arachidonic acid on SR function are better correlated with the ratio of arachidonic acid to protein (lipid) than with the concentration in solution suggests that the concentration achieved in the lipid phase of the membrane is the most important determinant of its action. Ischemia has been reported to double the concentration of arachidonic acid in the heart [2] from its resting level of 4.6 to 4.9 μmol/kg wet weight [2, 3] and to increase it even more after reperfusion (up to >60 μmol/kg wet weight [3, 44]).

If all the arachidonic acid were membrane associated and assuming that SR comprises 3% of the cardiac cell volume [45] and occupies a volume of 3 μL/mg protein [46], the resting arachidonic acid levels even during reperfusion would represent ~6 nmol/mg membrane protein in the membrane phase. This value is far below the 100 nmol/mg protein we estimate to be required to appreciably impair Ca^{2+} uptake from our isolated vesicle studies. It would seem unlikely that arachidonic acid levels in the heart would ever rise to the point where SR function would become severely impaired due to unmetabolized arachidonic acid alone. Indeed, Van Bilsen *et al.* [47] found no correlation between fatty acid content of hearts exposed to ischemia/reperfusion and indices of post-ischemic functional recovery. Whether the concentrations achieved would be likely to affect surface membrane K^+ channels [48] or gap junctions [43] remains to be ascertained.

The conclusion that arachidonic acid effects are modulated by the ratio of arachidonic acid to sample suggests that care needs to be taken in terms of any analysis of its effects extrapolated to intact tissue. For instance, a single isolated myocyte exposed to a large volume of a minimal concentration of arachidonic acid [e.g. see Ref. 48] would be far more likely to respond than would a whole heart exposed to the same concentration in a small volume. The partitioning of other lipophilic substances into membranes might also be likely to affect their actions. Consequently, greater caution should be exerted in extrapolating between effects of lipophilic substances on isolated cells or membranes in which the con-

centration of membranes relative to aqueous phases is vastly different from that encountered in the tissue from which those samples were derived.

This principle of efficacy related to concentration in the membrane phase is likely to apply to other lipid-soluble mediators of SR Ca^{2+} release, including palmityl carnitine [16], palmitoyl CoA [37], lysophospholipids [49], and oleate [50]. However, the other conclusions regarding the action of arachidonic acid drawn here have not been tested for their applicability to these other agents.

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