Methyl Palmitate: A Potent Vasodilator Released in the Retina

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PURPOSE. To determine whether palmitic acid methyl ester (PAME) or methyl palmitate is the retina-derived relaxing factor (RRF).

METHODS. A superfusion bioassay cascade technique was used with rat isolated retina as donor tissue and rat aortic ring as receptor tissue. The superfusate was analyzed with gas chromatography/mass spectrometry (GC/MS). The biochemical and pharmacologic characteristics of RRF and PAME were compared.

RESULTS. The authors demonstrated that the retina on superfusion with Krebs solution spontaneously released RRF (indicated by aortic ring relaxation) and PAME (measured by GC/MS). The release of RRF and PAME was calcium dependent because the release was abolished when the retinas were superfused with calcium-free Krebs solution. Furthermore, aortic relaxations induced by RRF and PAME were not affected after heating their solutions at 70°C for 1 hour, suggesting that both are heat stable. Exogenous PAME concentration dependent induced aortic relaxation with EC50 of 0.82 ± 0.75 pM. The aortic relaxations induced by RRF and exogenous PAME were inhibited by 4-aminoypyridine (2 mM) and tetraethylammonium (TEA, 10 mM) but were not affected by TEA at 1 mM or 3 mM, glibenclamide (3 μM), or iberiotoxin (100 nM). The vasodilator activity of Krebs solution containing RRF or exogenous PAME was greatly attenuated after hexane extraction.

CONCLUSIONS. RRF and PAME share similar biochemical properties and react similarly to all pharmacologic inhibitors examined. Both act primarily on the voltage-dependent K+ channel of aortic smooth muscle cells, causing aortic relaxation. These results suggest that PAME is the hydrophobic RRF. (Invest Ophthalmol Vis Sci. 2010;51:4746–4753) DOI:10.1167/iovs.09-5132

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The ophthalmic artery contributes the major blood supplies to the eye. It branches into a central retinal artery, short posterior ciliary arteries, and a nasal and temporal long posterior ciliary artery. The central retinal artery or cilioretinal arteries feed the retinal circulation, nourishing the inner two-thirds of the retina, and the ciliary arteries contribute to the choroidal circulation supplying the outer third of the retina. These two circulations exhibit very different properties. Whereas choroidal circulation has both sympathetic and parasympathetic innervations, the retinal circulation does not receive autonomic innervation. The retinal circulation is therefore thought to be regulated by autoregulatory mechanisms and local factors such as nitric oxide (NO), prostaglandins (PGs), epoxycosatrienoic acids (EETs), and many others released from neighboring cells.

Delaye and Van De Voorde in 1998 first reported a retina-derived relaxing factor (RRF), that was spontaneously released to relax the precontracted arteries obtained from several vascular beds. The RRF-induced relaxation is endothelium independent. Given that treatment of RRF-containing solution with trypsin does not alter its relaxing properties, RRF is unlikely a protein. Many known vasoactive molecules have been ruled out as candidates of RRF, including NO, prostanooids, adenosine, adenosine diphosphate, adenosine-5’-triphosphate, lactate, glutamate, γ-aminobutyric acid, taurine, adrenomedullin, calcitonin gene-related peptide, atrial natriuretic peptide, brain natriuretic peptide, and C-type natriuretic peptide. The identity of RRF remains unknown.

Using a superfusion bioassay cascade technique with rat superior cervical ganglion as donor tissue and rabbit endothelium-denuded aortic ring as detector tissue, we demonstrated for the first time that a potent vasodilator, palmitic acid methyl ester (PAME) or methyl palmitate, was released from the rat superior cervical ganglion on electrical depolarization. Here we further demonstrate that PAME also is released from the retina tissue and is likely the proposed RRF.

MATERIALS AND METHODS

Tissue Preparation

All experimental procedures were approved by the Laboratory Animal Care and Use Committee at Tzu Chi University and were in compliance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male Sprague-Dawley rats (350 - 450 g) were anesthetized with pentobarbital (60 mg/kg, intraperitoneally). The retinas and thoracic aorta were dissected and were placed in oxygenated (95% O2 and 5% CO2) Krebs solution at room temperature. Isolated retinas were incubated in oxygenated Krebs solution at 37°C in a Warburg incubator before use in the experiment. The thoracic aortic rings were stripped of perivascular adipose tissues (PVAT), and were mechanically denuded of endothelial cells (ECs). The successful removal of ECs was verified by lack of acetylcholine-induced relaxation.
Superfusion Bioassay Cascade Technique

A modified superfusion bioassay cascade system21 was used. Four retinas from two rats were placed in a 0.5-mL round centrifuge tube, of which the bottom was cut hollow and replaced with a nylon net. The retinas as donor tissues were superfused (3 mL/min) with oxygenated Krebs solution at 37°C through a peristaltic pump. The superfusate was allowed to superfuse an isolated rat aortic ring as detector tissue arranged in the cascade system. A simplified scheme of the superfusion bioassay cascade system is shown in Figure 1.

The rat aortic ring was used as the detector tissue because the rat aortic smooth muscle contains abundant α-adrenoreceptors readily producing consistent vasoconstriction by L-phenylephrine, which was delivered by a separate line (Fig. 1, line 1). Tension changes in the aortic ring were measured by using an isometric transducer (FT03C; Grass, West Warwick, RI) and were recorded on a Grass polygraph. A resting tension of 2 g was applied to the aortic ring. Varied concentrations of phenylephrine between 0.01 μM and 0.1 μM were applied to produce an active muscle tone of approximately 2 g in each arterial preparation.

In examining the effects of calcium-free Krebs solution on RRF or PAME release, a modified method was used as shown in Figure 1. Calcium-free Krebs solution, from superfusing the retinal preparation, was mixed with calcium-containing solution delivered from a separate line (line 2). Accordingly, the final Krebs solution before superfusion of the aortic ring contained a normal calcium concentration, allowing aortic constriction in the presence of phenylephrine delivered through line 1. Relaxation was estimated as percentage of sodium nitroprusside (SNP)-induced maximum relaxation. Perfusates, obtained after superfusing the retinal preparations and aortic rings, were collected and analyzed for PAME by gas chromatography/mass spectrometry (GC/MS).

Effect of Heated Krebs Solutions Containing RRF and PAME on Aortic Relaxation

Six retinas (wet weight, 16.2 ± 2.3 mg each) from three rats were incubated together in 1.5 mL oxygenated Krebs solution at 37°C for 1 hour. The incubation solutions were collected and centrifuged at 2000 rpm for 5 minutes. Half the supernatant was heated at 70°C for 1 hour. Heated and nonheated supernatants were applied onto the aortic ring precontracted with phenylephrine to examine whether heated RRF still caused vasodilation. In a parallel study, PAME (0.1 μM)-containing Krebs solution was heated at 70°C for 1 hour, and the aortic dilating effect of the heated Krebs solution was examined.

Effects of Hexane-Extracted RRF- and PAME-Containing Krebs Solutions on Aortic Relaxation

After incubation of retinal preparations in oxygenated Krebs solution at 37°C for 1 hour, as described in a previous study,13 3 mL supernatant was mixed with 3 mL (1:1 ratio) or 6 mL (2:1) hexane. The mixture was then vortexed for 20 minutes and centrifuged at 2000 rpm for 5 minutes, and the hexane fraction was discarded. This extraction with hexane was repeated two more times. Then the supernatant, with or without hexane extraction, was applied directly onto the precontracted aortic ring to examine whether hexane extraction removed the relaxing factor from the supernatant. In parallel, PAME was dissolved in Krebs solution with a final concentration of 0.1 μM. This PAME-containing Krebs solution was treated with hexane, as described, to determine whether hexane-extracted solution would no longer induce aortic relaxation.

Gas Chromatography/Mass Spectrometry Analysis

Perfusates from the superfusion bioassay cascade system were extracted with methanol to solubilize the organic compounds according to our previous report.21 The sample was vortexed, sonicated, and finally pelleted by centrifugation at 1500 rpm for 5 minutes at 20°C. The supernatant was transferred to screw-cap tubes with polytetrafluoroethylene/silicone septa in the caps. Samples were analyzed by using a gas chromatograph (GC; model 6890; Hewlett-Packard, Palo Alto, CA) equipped with an autosampler (G1521A; Hewlett-Packard) and interfaced to a mass selective detector (5975; Hewlett-Packard). The GC was equipped with a BPX5 5% phenyl polysilphenylene-siloxane capillary column (25 m × ID 0.22 mm; film thickness, 0.25 μm). Helium, at a flow rate of 0.6 mL/min, was the carrier gas. Temperatures for the GC injection port and interface were maintained at 250°C and 300°C, respectively. The GC temperature started at 90°C, increased 15°C/min to 240°C, 10°C/min to 300°C. The mass spectrum was obtained by scanning from m/z 50 to 550. The mass spectrometer was operated in electron impact mode at electron ionization energy of 70 eV. Splitless injection mode was used with an injection volume of 2 μL. Software (ChemStation G1701AA version 0.300; Hewlett-Packard) in the drug analysis mode was used for data acquisition and analysis.

Chemicals and Solutions

Krebs solution consisted of NaCl 117 mM, NaHCO3 25 mM, KCl 4.7 mM, CaCl2 2.5 mM, MgSO4 1.2 mM, KH2SO4 1.2 mM, glucose 11.1 mM, and ascorbic acid 0.28 mM. In calcium-free Krebs solution, 2.5 mM CaCl2 was removed without substitution. Phenylephrine hydrochloride, SNP, PAME, methyl stearate or stearic acid methyl ester (SAME), L-N^ nitroarginine (L-NNA), indomethacin, miconazole, SKF525A, and tetraethylammonium (TEA) were from Sigma-Aldrich; glibenclamide and iberiotoxin were from Tocris (Ellisville, MO), and 4-aminopyridine (4-AP) was from Fluka (St. Louis, MO); hexane was from J.T. Baker (Phillipsburg, NJ). PAME was dissolved in absolute methanol and then diluted with Krebs solution to a final methanol concentration of <0.1% vol/vol. L-NNA was dissolved in acidic normal saline; indomethacin, miconazole, 4-AP, and glibenclamide were dissolved in dimethyl sulfoxide, and SNP, TEA, iberiotoxin and SKF525A were dissolved in distilled water.

Statistical Analysis

Data were expressed as mean ± SEM. One-way ANOVA and paired t-test were used to determine effects of calcium, indomethacin, or...
L-NNA on RRF release and those of the heat- or hexane-treated RRF- or PAME-containing Krebs solution on their vasodilatory activities. In these experiments, control and experimental studies were performed in a continuous perfusion manner. Two-sample t-test was used to compare the effects of TEA, 4-AP, glibenclamide, iberiotoxin, SKF525A, and miconazole on retinal or aortic preparations. Nonlinear (sigmoid) regression was used to estimate the concentration-response relationship and EC50 values. Statistical software (OriginPro 7.5; OriginLab Corporation, Northampton, MA) was used for statistical analysis. P < 0.05 was considered statistically significant.

RESULTS

Spontaneous Release of RRF from the Isolated Retinal Preparations

In the presence of active muscle tone induced by L-phenylephrine (0.1 μM), the aortic rings (without EC and PVAT) as detector tissues down the cascade were relaxed by normal Krebs perfusates after superfusion of the retina (donor tissue; Fig. 2). This aortic relaxation indicates spontaneous release of a transferable RRF. This relaxation was immediately converted to a constriction toward its original level of active muscle tone on removing the retina (donor tissue) away from the perfusion cascade (i.e., in the absence of RRF) (Fig. 2). The spontaneous release of RRF, as indicated by the aortic relaxation, was repeatable (Fig. 2).

Spontaneous Release of PAME from the Isolated Retinal Preparations

The perfusates, after superfusing the retinas and causing relaxation of aortic rings, were collected for GC/MS analysis of PAME. Two peaks of the GC/MS were identical with those of PAME (retention time, 12.39 minutes) and SAME (retention time, 13.75 minutes; Fig. 3A). Two peaks of the mass spectrometry analysis matched the library ID of PAME (Fig. 3B) and SAME (Fig. 3C), respectively.

PAME as a Potent Vasodilator

In the presence of active muscle tone induced by L-phenylephrine, aortic rings (without EC and PVAT) relaxed on application of exogenous PAME in a concentration-dependent manner. The Emax value was 51.2% ± 3.1% (of SNP-induced maximum relaxation), and the EC50 value was 0.82 ± 0.75 pM (Fig. 4). SNP applied directly onto the phenylephrine-precontracted aortic rings also induced a concentration-dependent relaxation (data not shown). The Emax of SNP was 100%, and the EC50 value was 360 ± 190 pM.

Calcium Dependent Release of RRF and PAME

To examine whether calcium in the Krebs solution is critical for the spontaneous release of RRF and PAME from the retina,
nearly abolished aortic relaxations induced by PAME (0.1 μM; 92.4% and 92.6%) and RRF and PAME by 90.7% (Fig. 5). PAME release from the retinal preparations was almost abolished when calcium-free Krebs solution was used (Fig. 5).

Parallel Reduction of Aortic Relaxation Induced by RRF- and PAME-Containing Krebs Solutions after Hexane Extractions

The Krebs solutions containing RRF released from the retinal preparations after incubation at 37°C for 1 hour and those containing exogenous PAME (0.1 μM) were subjected to hexane extractions. After equal volumes of hexane extractions (3 mL Krebs solution extracted with 3 mL hexane, or a 1:1 ratio) repeated three times, both RRF- and PAME-containing Krebs solution-induced aortic relaxations were significantly decreased by 40.21% ± 9.42% and 38.10% ± 9.48%, respectively, of their controls (Fig. 8B), and there was no PAME detectable in either Krebs solution as determined by GC/MS (data not shown). Residual relaxations induced by both were almost abolished by 4-AP (2 mM, Fig. 8B). After extractions of Krebs solutions containing RFF and PAME with double volumes of

L-NNA and Indomethacin Potentiation of Aortic Relaxation Induced by RRF and PAME

When the relaxation of aortic rings induced by Krebs perfusates after superfusion of the retina (donor tissue) almost reached the maximum or leveled off, the addition of L-NNA (100 μM; Figs. 7A, 7B) or indomethacin (10 μM; Figs. 7C, 7D) to the retinal preparations further enhanced relaxation of the aortic rings. Addition of L-NNA and indomethacin directly onto aortic rings did not affect the Krebs superfusion of retinal-induced aortic relaxation or that induced by exogenous PAME (data not shown).

Failure of SKF525A and Miconazole, Inhibitors of EET Synthesis, to Affect Aortic Relaxation Induced by RRF or PAME

When the relaxation of aortic rings (indicative of RRF release) induced by Krebs perfusates after superfusion of the retina almost reached maximum or leveled off, the addition of SKF525A (10 μM) or miconazole (60 μM) to the retina did not significantly affect relaxation of the aortic ring (Figs. 7E, 7F). Similarly, SKF525A or miconazole at the same concentrations superfused directly onto the aortic ring did not affect the aortic relaxation induced by exogenous PAME added directly onto the aortic rings (data not shown).

Failure of Heating to Affect RRF- or PAME-Induced Vasodilation

After retinal preparations were incubated in Krebs solution at 37°C for 1 hour, this Krebs solution induced aortic relaxation, indicative of the presence of RRF (Fig. 8A). Krebs solution containing PAME (0.1 μM) applied directly onto the aortic rings also induced aortic relaxation. Aortic relaxations induced by both RRF- and PAME-containing Krebs solutions that had been heated at 70°C for 1 hour were not different from the controls (Fig. 8A).
hexane (3 mL Krebs solution extracted with 6 mL hexane, or a 2:1 ratio) repeated three times, the aortic relaxations were almost abolished (Fig. 8C).

**DISCUSSION**

Results of the present study provide evidence for the first time that PAME is likely the RRF. This is based on findings of similar chemical properties and identical modes of action of PAME and RRF. The findings are that PAME and RRF are spontaneously released from the isolated retinal preparations; PAME is a potent vasodilator; the releases of RRF and PAME are totally calcium dependent; both RRF- and PAME-induced aortic relaxations are blocked specifically by inhibitors of voltage-dependent K[\text{v}] channels; vasorelaxant activities of RRF or PAME are not affected after heat treatment at 70°C; and the aortic relaxation induced by Krebs solution containing PAME or RRF is abolished or reduced to a similar extent after hexane extractions.

Our findings also indicate that NO, PG, and EET are not the RRF. This is consistent with the reports by others.16,17,20 These authors, using a standard submerging tissue bath technique, demonstrated that L-NNA (a NO-synthase inhibitor) and indomethacin and sodium diclofenac (COX inhibitors) failed to block the dilation of isolated rat carotid arteries or mouse aorta induced by placing rat or mouse retinal preparations immediately on top of these arteries.16,17 COX inhibitors, in fact, enhanced the retina-induced vasodilation. This is consistent with results of the present study that indomethacin and L-NNA superfused directly onto the retina enhanced aortic relaxation down the cascade, whereas both inhibitors superfused directly onto the aortic ring did not affect the RRF-induced aortic relaxation. Similarly, SKF-525A and miconazole (EET synthase inhibitors) did not affect retina-induced aortic relaxation, indicating that EET is not likely the RRF.

The potent vasodilating effect of PAME21 prompted us to speculate that PAME was the RRF. The high potency of PAME in inducing vasodilation is also supported by results of the present study. The calculated EC50 value for PAME in inducing aortic relaxation is in pM ranges and is at least 400 more potent than NO, making it the most potent known vasodilator. Lack of information on the biosynthesis and metabolism of PAME, however, prevented our using molecular strategies to determine whether PAME is the RRF. Alternative strategies by comparing chemical and pharmacologic properties, therefore, were used. The results strongly favor the hypothesis that PAME is the RRF for several reasons.

First, both RRF and PAME releases are calcium dependent. Our previous study demonstrated that the release of PAME from the rat superior cervical ganglion was calcium dependent.21 In the present study, retinal preparations superfused with calcium-free Krebs solution failed to cause relaxation of the detector aortic rings, suggesting the absence of RRF release. This was accompanied by the diminished release of endogenous PAME (determined by GC/MS) from the retinal preparations. When calcium-free Krebs solution was replaced by calcium-containing Krebs to superfuse the same retinal preparations, the release of RRF as indicated by aortic relax-
ation and the release of PAME were observed. These results indicate that the release of both RRF and PAME from the retinal preparations is calcium dependent. Like that found in the superior cervical ganglion of the rat,21 the present finding indicated that SAME was released together with PAME from the retina, and the release was also calcium dependent. SAME, however, did not cause aortic relaxation, a result similar to our previous findings.21 The exact functional role of SAME remains undetermined.

Second, both RRF and PAME induced aortic relaxation by acting on the voltage-dependent potassium (Kv) channel located on the smooth muscle cells. Others have shown that the RRF relaxes arteries precontracted with PGF2α, U-46619, serotonin, and endothelin-1, but it induces small relaxation in arteries precontracted with high concentrations of K+ (120 mM).15 These findings suggest that the RRF-induced vasorelaxation may involve opening of the K+ channels on the vascular smooth muscle cells. In the present study, aortic relaxations induced by RRF and exogenous PAME were inhibited in parallel by 2 mM 4-AP (a specific inhibitor for the Kv channel24–28) and 10 mM TEA (a nonspecific inhibitor for calcium-activated potassium channels, voltage-dependent K+ channels, and ATP-sensitive K+ channel/KATP24). Aortic relaxations induced by RRF and exogenous PAME, however, were not affected by lower concentrations of TEA (1 mM or 3 mM; a preferential inhibitor for the calcium-activated potassium channel24–28), glibenclamide (a KATP channel inhibitor24–25), or iberiotoxin (a specific inhibitor for the calcium-activated potassium channel24–28). These findings suggest that the RRF- and PAME-induced aortic relaxations are similarly mediated by opening the voltage-dependent K+ (Kv) channels on the smooth muscle cells. Given that EC and PVAT have been shown to release vasodilating substances,22,29–32 results of the present study using aortic ring without EC and PVAT further support the direct action of RRF and PAME on the muscle cells.

Third, both RRF and PAME are transferable and heat stable. Aortic relaxations induced by RRF and PAME were not appreciably affected after their Krebs solutions were heated at 70°C for 1 hour. These results are consistent with those reported by Delaey and Van de Voorde15 indicating that RRF is heat stable.

Fourth, there were parallel reductions of aortic relaxation induced by hexane-extracted RRF- and PAME-containing Krebs solutions. It was reported by Delaey and Van de Voorde15 that vasorelaxation induced by bovine RRF-containing Krebs solutions after subjection to 1:1 hexane extractions repeated three times was not significantly different, though a slight decrease in vasodilation appeared to occur compared with that induced by RRF-containing solutions before hexane extractions.15 These authors therefore concluded that RRF appeared hydrophilic in nature. However, in the present study, rat RRF- and PAME-containing Krebs solutions, after being subjected to repeated 1:1 hexane extractions, induced parallel reductions of aortic relaxation compared with those induced by solutions before hexane extractions. The residual relaxations were almost abolished by 4-AP (2 mM), not only confirming the presence of residual PAME in hexane-extracted PAME-containing Krebs solutions but also suggesting the presence of PAME-like substance in RRF-containing Krebs solution.

**FIGURE 7.** Effects of L-NNA, indomethacin, SKF525A, and miconazole on RRF- induced aortic relaxation. Representative tracings indicate that aortic relaxation after Krebs solution superfusion of the retinal preparation (+) was enhanced by L-NNA (A) and indomethacin (C) applied directly onto the retina. Their summaries are shown in (B) (P < 0.001) and (D) (P < 0.05), respectively. This aortic relaxation, however, was not affected by SKF525A (E) or miconazole (F) applied directly onto the retina. Data are mean ± SEM. n = number of independent experiments.
It should be noted in the present study that in Krebs solutions containing RRF and PAME (0.1 μM)-containing Krebs solutions were not affected after heating both solutions at 70°C for 1 hour (A), but they were equally and significantly reduced after equal volumes of hexane (1:1) extractions three times (B, C). The remaining relaxations induced by both were further inhibited by 4-AP (B) applied directly onto the aortic rings, or solutions were subjected to double volume of hexane (2:1) extractions (C). $n =$ number of experiments. (B, C) $^* P < 0.05$, hexane extracted (1:1) versus control; (B) $^{a P} < 0.05$, 4-AP versus hexane extracted (1:1); (C) $^{a P} < 0.05$, hexane extracted (2:1) versus hexane extracted (1:1).

These latter findings suggest that 1:1 hexane extractions did not completely remove PAME in the Krebs solutions. Any remaining minute amounts of PAME, which were beyond the detecting capacity of GC/MS, can still cause vasorelaxation. This is highly possible because PAME is extremely potent, as shown in our previous studies and the present study. Furthermore, after subjecting RRF or PAME to repeated extractions with double volume hexane (2:1), the aortic vasorelaxations induced by these solutions were almost abolished. These results suggest that RRF behaves like PAME and is hydrophobic, though species variation cannot be excluded.

An important concern in the bioassay of hydrophobic vasoactive substances such as PAME is the inappropriate use of submerged tissue bath technique, since the vasodilatory property of PAME is difficult to detect using this technique. This is due to the fact that on application of exogenous PAME or other hydrophobic fatty acids which are dissolved in methanol stock solution, these fatty acids quickly come out of solution as soon as they are added to a “larger” volume of the Krebs solution in the tissue bath. Accordingly, these fatty acids will not reach the submerged tissues under examination. This problem is avoided by the use of the superfusion bioassay cascade system in the present study, which enables detection of biological activity of the hydrophobic substances by directly perfusing them onto the aortic rings to cause a response (Fig. 1). Thus, the submerged tissue bath technique used by Delaey and Van de Voorde (1998) might limit them from a full evaluation of possible hydrophobic compounds. Our present studies, therefore, have provided strong evidence suggesting that PAME is a hydrophobic RRF.

It will be interesting and important to demonstrate that “RRF” and methyl palmitate relax the retina arterioles which may have different pharmacologic properties from large arteries. Use of retinal arteries as the detector tissue for bioassay for RRF, however, is technically difficult due to the small size and diameter of the artery. The retinal arteriolar smooth muscle cells, however, are endowed with 4-AP-sensitive voltage-dependent Kv channels. It is likely that RRF and methyl palmitate will relax these arterioles.

In summary, both RRF and PAME induced endothelium-independent aortic relaxation. The mode of action and pharmacologic properties of RRF found in the present study are similar to a large extent to those reported by others. We, however, for the first time, demonstrate that RRF behaves almost identically with PAME. Both RRF and PAME are heat stable, hydrophobic, and act on Kv channels on the smooth muscle cells to induce vasorelaxation. Several pharmacologic blockers, which failed to affect relaxation induced by RRF, did not affect that induced by PAME. In addition, releases of both RRF and PAME are totally calcium dependent. These identical biochemical and pharmacologic characteristics of RRF and PAME strongly favor the hypothesis that PAME is the hydrophobic RRF.

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**References**


