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Extracellular and intracellular arachidonic acid-induced contractions in rat aorta

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Abstract

Arachidonic acid induced contractions of de-endothelized rat aortic rings. A more potent effect was obtained after intracellular administration of arachidonic acid using liposomes. Contractions induced by extracellular arachidonic acid were inhibited similarly to phenylephrine-induced contractions by the L-type Ca^{2+} channel blocker, methoxyverapamil (D600), and the calmodulin inhibitor, calmidazolium. In contrast, contractions induced by arachidonic acid-filled liposomes were not affected by these compounds. Indomethacin did not affect the contractions induced by either extra- or intracellular arachidonic acid, whereas nordihydroguaiaretic acid relaxed contractions induced by extracellular arachidonic acid but not those induced by arachidonic acid-filled liposomes. Apart from a relaxing effect on contractions induced by extracellular arachidonic acid or by phenylephrine, protein kinase C inhibition with 1-(5-isoquinolinesulphonyl-2-methylpiperazine (H7)) had an even more prominent relaxing effect on contractions induced by arachidonic acid-filled liposomes. Therefore, arachidonic acid exerts a contractile effect on rat aorta, and this effect is regulated differently depending on the site of application. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Arachidonic acid; Smooth muscle; Aorta; Contraction; Liposome

1. Introduction

Formation of arachidonic acid from membrane phospholipids can occur either by activation of phospholipase A_2 or a sequential action of phospholipase C and diglyceride lipase (Axelrod, 1990). Many smooth muscle membrane receptors are coupled to activate these mechanisms (Horowitz et al., 1996). As a consequence, contractile agents induce increases in arachidonic acid concentration. Changes in arachidonic acid concentration were reported to occur in certain pathological states, such as cardiovascular and brain ischemia (Katsura et al., 1993; Oe et al., 1994) or neurotrauma (Bazan et al., 1995), with immediate impact on survival of the damaged cells. Also, in other cell types, arachidonic acid is implicated in physiological functions such as hormone release (Roudbarski et al., 1995).

The importance of arachidonic acid in cellular signal transduction has been strongly reevaluated in the last decade. It was demonstrated that arachidonic acid has a multitude of cellular effects, supporting its role as second messenger in smooth muscle. Arachidonic acid can release Ca^{2+} from intracellular stores (Fleming and Mellow, 1995; Vacher et al., 1992), stimulates Ca^{2+} entry independent of inositol phosphate formation (van der Zee et al., 1995), inhibits voltage-dependent Ca^{2+} entry (Shimada and Somlyo, 1992), and sensitizes the smooth muscle contractile apparatus to basal Ca^{2+} concentrations (Gong et al., 1992). Further, arachidonic acid activates protein kinase C (Lester et al., 1991), inhibits K^+ conductance (Soliven and Wang, 1995) and is able to uncouple gap junctions (Lazrak et al., 1994). In a number of non-smooth muscle cells, arachidonic acid inhibits a store-dependent capacitative Ca^{2+} influx (Gamberucci et al., 1997) and the mobilization of arachidonic acid is coupled to store depletion and Ca^{2+} influx (Rzigalinski et al., 1996). The majority of the above-cited effects are mediated solely by arachidonic acid and not by its metabolic products.

The effects of arachidonic acid are predominantly studied by extracellular administration or in permeabilized

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preparations. Because formation occurs intracellularly before it is released extracellularly, it is essential to study intracellular effects of arachidonic acid in intact preparations. We previously showed that liposomes are an ideal tool for intracellular delivery of various compounds, as the cellular membrane remains intact during the process (Brailoiu et al., 1993, Brailoiu and van der Kloot, 1996). The aim of the present work was to compare the effects of extracellularly applied arachidonic acid and those of intracellularly applied (using liposomes) arachidonic acid, in rat aorta vascular smooth muscle. The observations demonstrate that under both conditions arachidonic acid induces contraction, but by different mechanisms.

2. Material and methods

2.1. Tissue preparation

Male Wistar rats weighing 175–250 g (12–14 weeks old) were killed by cervical dislocation and exsanguinated. The descending thoracic aorta was carefully removed and immersed in modified Krebs Henseleit solution (physiological salt solution, PSS) with the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.6, $NaHCO_3$ 24.9, EDTA 0.03, glucose 5.55 (pH 7.3 adjusted with NaOH). The aorta was dissected free of the surrounding connective tissue and cut into 2-mm-wide segments. The averaged wet weight of rat aortic rings amounted 1.6 ± 0.05 mg ($n = 6$). The endothelium was removed by gentle rubbing with a smooth softwood stick.

2.2. Experimental procedures

Each ring was mounted vertically between a pair of stainless-steel wire hooks in 2-ml organ baths containing PSS warmed at 37°C and continuously aerated with 95% O_2 and 5% CO_2. The upper hook was attached to an isometric force transducer type IMF 002. The electrical signal was converted to digital data by a Keithley A/D 2000 board and recorder by a PC 486 using a data acquisition program (PeakExe), as described earlier (Filipeanu et al., 1997).

An initial tension of 2 g was imposed on each ring. After an equilibration period of 90 min (bath medium changed every 15 min), the preparations were stimulated repeatedly with 10 μM phenylephrine, until two consecutive contractions with an amplitude differing by less than 5% were obtained. In the plateau of these contractions, the successful removal of endothelium was tested as absence of relaxation in response to 10 μM carbachol. The amplitude of the last contraction was considered as 100% for further comparison. The rings that did not develop 0.8 g active force were discarded.

After re-equilibration of the preparations the desired drug was added in the bath from 100 times more concentrated solutions. To maximize the contractile effect, liposome batches (0.5 ml) were added to the 2-ml organ bath containing 1.5 ml PSS (Brailoiu et al., 1995). Arachidonic acid was added only once to each preparation to avoid desensitization. Relaxing agents were added on the plateau of contraction. Only after stabilization of the response (10 min without variations in force greater than 1% from the amplitude of total contraction) was a further addition performed.

2.3. Liposomes preparation

The liposomes used in these physiological studies were prepared from egg phosphatidylcholine (type X-E; Sigma), 60 mg lipid/ml of solution to be incorporated, according to the method described by Bangham et al. (1965) as modified by Brailoiu et al. (1993). Control liposomes contained only KCl (140 mM, pH adjusted to 6.9). Liposomes containing different amounts of arachidonic acid were also prepared in this manner, but arachidonic acid dissolved in dimethylsulfoxide was included. The final solutions from which the liposomes were prepared ranged from 0.1 to 200 μM arachidonic acid, containing 0.0004–0.8% dimetylsulfoxide. After mechanical agitation the suspensions were treated with diethylether in a 1/10 (v/v) ratio. Before thin layer chromatography (TLC) studies or contractile experiments, the solvents (diethylether and dimethylsulfoxide) were removed under reduced pressure, or by dialysis in PSS (150 min, 1/600 (v/v) ratio with exchange of buffer every 30 min), respectively.

2.4. Thin layer chromatography analysis

In order to test vesicle integrity, thin layer chromatography was performed exactly as described previously (Brailoiu et al., 1995). The liposomes were applied to the TLC plates at equal amounts of lipids. The solvent used for analysis contained *n*-butanol/ethanol/water in a 4/3/3 volume ratio. The time for running was 45 min in all cases. Visualisation was performed with a Dragendorffs spray reagent for nitrogen-containing compounds. The differences between the migration of liposome batches were obtained from the relative ratio (R_f) between the distance of run migration and the distance of front migration.

2.5. Determination of the arachidonic acid concentration delivered into rat aorta smooth muscle cells

The liposomes were prepared exactly as described above, but [5,6,8,9,11,12,14,15-^{3}H(N)]arachidonic acid (specific activity 186.4 Ci/mmol) was dissolved in the aqueous phase. After dialysis, the liposomes were incubated with rat aortic rings for 10 min in PSS solution at 37°C. At the end of this period, the rings were washed five times with PSS solution at room temperature in order to remove all possible extracellular radioactivity. After that,

the rings were dissolved overnight in 0.5 M NaOH and their radioactivity determined by liquid scintillation counting. The radioactivity of the last three washes was at least 10 times less than the radioactivity of the rings, excluding a contribution of extracellular radioactivity to the results.

2.6. Drugs

Phenylephrine, arachidonic acid (5,8,11,14-eicosotetranoic acid), nordihydroguiaretic acid, H7 (1-(5-isoquinolinesulphonyl-2-methylpiperazine), calmidazolium and phosphatidylcholine (type XE) were obtained from Sigma, D600 (methoxyverapamil) from Knoll, and [5,6,8,9,11,12,14,15-^{3}H(N)]arachidonic acid from Dupont-NEN products.

Arachidonic acid was dissolved in dimethylsulfoxide as a stock solution of 10 mM and stored at −20°C until use. Calmidazolium stock solution was prepared in distilled water at a concentration of 1 mM and kept at 4°C. All other drugs were dissolved in distilled water on the day of the experiment.

2.7. Statistics

All series were performed in at least four different animals ($n \geq 4$). The unpaired Student's t-test was used, with $P < 0.05$ considered significantly different.

3. Results

Thin layer chromatography showed that there were significant differences in migration only between control liposomes and liposomes prepared from solutions containing arachidonic acid in concentrations above 100 μM (Table 1). This means that these liposomes are able to deliver their content into the cells in a dose-dependent manner (Brailoiu et al., 1995). Therefore, no experiments were performed with liposomes prepared from arachidonic acid concentrations above 100 μM.

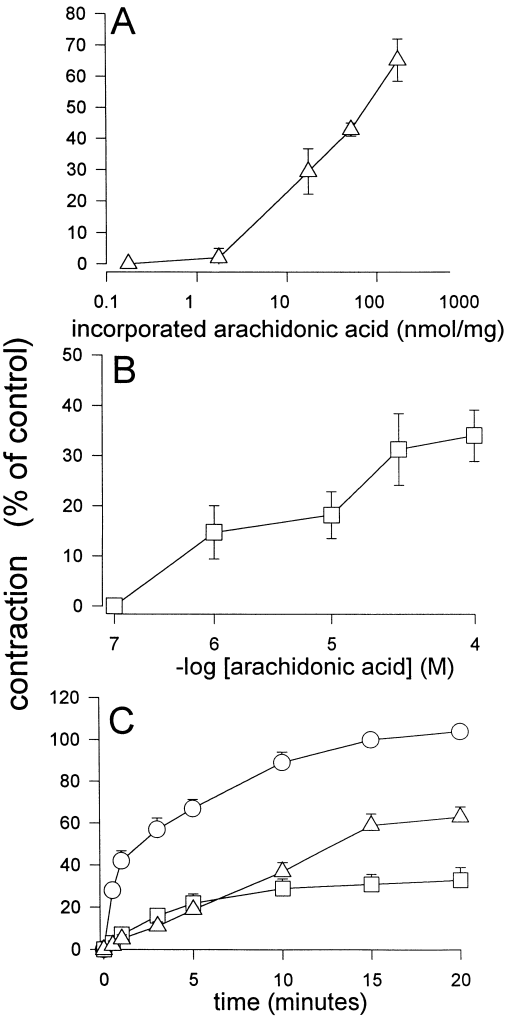

Fig. 1. Contraction of de-endothelized rat aorta induced by intracellularly delivered arachidonic acid and extracellularly applied arachidonic acid. (A) Dose–effect curve for different amounts delivered intracellularly using arachidonic acid-containing liposomes. (B) Dose–effect curve for extracellularly applied arachidonic acid. (C) Time dependence of the contractile response for phenylephrine (○, 10 μM), extracellularly applied arachidonic acid (□, 100 μM) and intracellular arachidonic acid application (△, liposomes prepared from 100 μM arachidonic acid solution). Data presented as means ± S.E.M., n = 4–10 in each case and normalized to the maximal contraction obtained with phenylephrine (10 μM).

Table 1
Relative mobilities of arachidonic acid-filled liposomes after TLC

Arachidonic acid concentration	Rf value
Control	19.4 ± 0.4
0.1 μM	19.4 ± 0.3
1 μM	19.2 ± 0.2
10 μM	19.3 ± 0.9
100 μM	20.2 ± 0.4
200 μM	16.1 ± 0.4[a]

The relative mobilities (Rf values) presented as the ratio between the distance of run migration and the distance of front migration for liposomes filled with different concentrations of arachidonic acid. Data are expressed as means ± S.E.M. (n = 6 in each case).
[a] Significantly different from control values.

Radioactivity determination showed that 53 ± 2% (n = 5) of the initial amount of [^{3}H]arachidonic acid in the aqueous solution was entrapped in the liposomes. Rings treated with these liposomes incorporated 0.28 ± 0.06% (n = 5) of the initial amount of arachidonic acid, corresponding to an intracellularly delivered amount of arachidonic acid ranging from 0.28 to 280 nmol/mg of wet tissue, using liposomes prepared from arachidonic acid concentrations between 0.1 and 100 μM. Addition of these batches of arachidonic acid-filled liposomes induced dose-dependent contraction of rat aorta rings (Fig. 1A,C). Application of control liposomes filled with 140 mM KCl or the dialysis medium of the liposomes did not modify the tonus

of relaxed or contracted preparations ($n = 10$, data not shown).

A contractile effect was also obtained after extracellular application of arachidonic acid (Fig. 1B,C). The time necessary for the development of the contractile response was longer for the arachidonic acid-filled liposomes (Fig. 1C). Both types of contractions (extracellular and intraliposomal arachidonic acid-induced) were abolished in Ca^{2+}-free PSS (data not shown).

In order to gain an insight into the mechanism involved in arachidonic acid-induced rat aorta contractions, the effects were tested of the voltage-dependent Ca^{2+} channel blocker, D600. An optimal concentration of D600 to block voltage-dependent Ca^{2+} channels in rat aorta (10 μM D600, Marriott, 1988) significantly inhibited the contraction elicited by 100 μM extracellular arachidonic acid by $28 \pm 5\%$; $P = 0.002$ (Fig. 2). This relaxation was almost similar to that induced by D600 on the plateau of phenylephrine-induced rat aorta contraction (inhibition with $35 \pm 4\%$; $P = 0.001$ (Fig. 2). In contrast, D600 was ineffective (change of $6 \pm 2\%$) to inhibit the contractile plateau induced by arachidonic acid-filled liposomes, which was significantly different from the effects of extracellular arachidonic acid ($P = 0.017$; Fig. 2).

A similar pattern was observed for relaxations induced by the calmodulin inhibitor, calmidazolium. As shown in Fig. 3A, this compound relaxed to a similar extent rat aorta contractions elicited by phenyleprine or by extracellular arachidonic acid, but was without effect on the contractile plateau induced by arachidonic acid-filled liposomes.

Arachidonic acid can be metabolized into different active compounds via the cyclooxygenase and lipoxygenase pathways. Inhibitors of these pathways were used to test

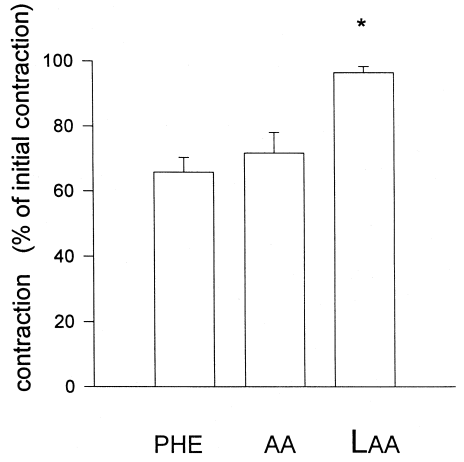

Fig. 2. Effect of D600 on contraction induced by phenylephrine, extracellularly applied arachidonic acid, and intracellularly applied arachidonic acid. The values are normalized to the maximal contraction obtainable with the respective contractile agent. PHE: phenylephrine (10 μM); AA: extracellular arachidonic acid application (100 μM); LAA: intracellular arachidonic acid application (liposomes prepared from 100 μM arachidonic acid solution). Data presented as means ± S.E.M., $n = 5$. Significance level $* P < 0.05$ in comparison with AA and PHE.

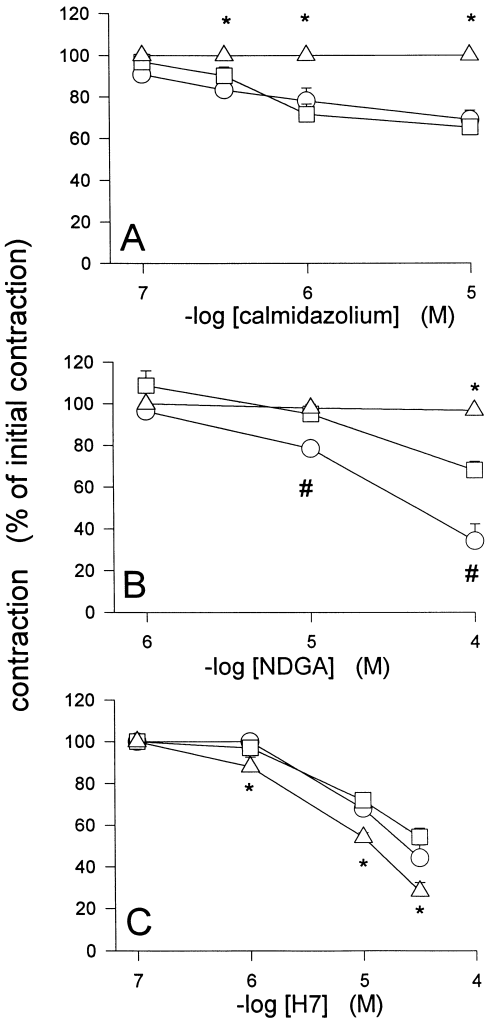

Fig. 3. Effect of various agents on contraction induced by phenylephrine, extracellularly applied arachidonic acid or intracellularly applied arachidonic acid. Concentration–effect curves for (A) calmidazolium, (B) nordihydroguaiaretic acid (NDGA), (C) H7. Data are normalized as % of the initial contraction of the respective contractile agents and presented as means ± S.E.M., $n = 4$–6. Phenylephrine (○, 10 μM); extracellularly applied arachidonic acid (□, 100 μM); intracellular arachidonic acid application (△, liposomes prepared from 100 μM arachidonic acid solution). Significance level $* P < 0.05$ for arachidonic-filled liposomes in comparison with the other two series. $^{\#}P < 0.05$ for phenylephrine in comparison with the other two series.

their involvement in the arachidonic acid-induced contractile responses. Indomethacin (30 μM) at an effective concentration for cyclooxygenase inhibition, affected neither contractions induced by extracellular ($102 \pm 3\%$, $n = 4$) nor those induced by intraliposomal arachidonic acid (98 ± 6, $n = 4$). At the higher concentrations used, the inhibitor of the lipoxygenase pathways, nordihydroguaiaretic acid, relaxed contractions induced by extracellular arachidonic acid to $68 \pm 4\%$ ($P = 0.032$ at 100 μM; Fig. 3B). Contractions induced by arachidonic acid-filled liposomes were insensitive to the relaxing effects of nordihydroguaiaretic acid. Nordihydroguaiaretic acid was even

more potent to relax phenylephrine-induced contractions (Fig. 3B), which could be attributed to a direct inhibitory effect of nordihydroguaiaretic acid on voltage-dependent Ca^{2+} channels (Korn and Horn, 1990). Consistent with this, we observed no further relaxation of phenylephrine-induced contractions if D600 was applied after nordihydroguaiaretic acid treatment ($3 \pm 4\%$, $n = 5$).

Finally, we tested the involvement of protein kinase C in the contractions induced by arachidonic acid. To this end we used the protein kinase C inhibitor, H7. As shown in Fig. 3C, the contractions induced by arachidonic acid-filled liposomes were strongly inhibited by this compound. The contractions induced by extracellular arachidonic acid and phenylephrine were also inhibited, but to a significantly lesser extent ($P = 0.031$ for intraliposomal arachidonic acid vs. extracellular arachidonic acid).

4. Discussion

Growing experimental evidence supports an important role of arachidonic acid in cellular signal transduction. Arachidonic acid can be formed by at least three different mechanisms: (1) activation of phospholipase A_2, (2) activation of diacylglycerol lipase hydrolysing diacylglycerol generated after induction of phospholipase C from phosphatidylinositol or phosphatidylcholine and (3) diacylglycerol lipase activity on phosphatidic acid formed after stimulation of phospholipase D (Axelrod, 90). Receptor-mediated contraction of vascular smooth muscle is known to be positively coupled with these mechanisms, and induces increases in the arachidonic acid levels (Horowitz et al., 1996). Since arachidonic acid formation can occur at the internal face of the cellular membrane and thereafter be released in the extracellular space (Khan et al., 1995), it is important to compare the effects induced by extracellular arachidonic acid with those induced intracellularly. In order to investigate these differences, we now used the liposome technique, which has been demonstrated to allow intracellular drug delivery (Brailoiu et al., 1993; Brailoiu and van der Kloot, 1996). Increasing the liposomal arachidonic acid content, without affecting the integrity of the liposomal membrane, enabled us to study the dose dependence of the effect of intracellularly delivered arachidonic acid (Brailoiu et al., 1995).

Apparently, different mechanisms are involved in the contractile action of arachidonic acid depending on which side of the plasma membrane it is presented. Intracellular arachidonic acid induced a higher contraction in rat aorta than did extracellular application. Further, pharmacologically active agents modulated the contractions induced by arachidonic acid-filled liposomes differently from those elicited by extracellular application of arachidonic acid. These latter contractions were inhibited to the same extent as those in response to the α_1-adrenoceptor agonist, phenylephrine, by the Ca^{2+} channel blocker, D600, and the calmodulin antagonist, calmidazolium. This suggests that extracellular arachidonic acid-induced contraction has the characteristics of agonist-induced contraction in rat aorta, except for its occurrence in Ca^{2+}-free medium. In contrast, contractions induced by arachidonic acid-filled liposomes were almost insensitive to calmidazolium and to Ca^{2+} entry via channels sensitive to D600, but the presence of extracellular Ca^{2+} is also needed to induce this type of contraction. This observation precludes the involvement of the classical smooth muscle contraction mechanism based on Ca^{2+} entry from the extracellular space via voltage-dependent channels and calmodulin-dependent activation of myosin light chain kinase (Horowitz et al., 1996).

Arachidonic acid metabolites are known to influence actin reorganization and mediate growth factor-induced cytoskeletal changes (Peppelenbosch et al., 1993). Participation of these metabolites in the effects of arachidonic acid-filled liposomes can be excluded, because inhibition of the cyclooxygenase and lipoxygenase pathways with indomethacin and nordihydroguaiaretic acid did not affect contraction. Indomethacin was also without effect on extracellular arachidonic acid-induced contractions, whereas under these conditions lipoxygenase products may account for about 30% of the total contractile response. It is more likely that this part can be attributed to the nonspecific effect of nordihydroguiaretic acid on voltage-dependent Ca^{2+} channels (Korn and Horn, 1990), because D600 failed to further relax phenylephrine-induced contractions after nordihydroguaiaretic acid and a similar sensitivity to D600 was observed for extracellular arachidonic acid- and for phenylephrine-mediated contractions.

Protein kinase C activation plays a role in smooth muscle contraction (Chiu et al., 1987; Rasmussen et al., 1987). Among the various compounds tested, as described, only the nonselective protein kinase C inhibitor, H7, had a strong relaxing effect on arachidonic acid-filled liposome-induced contractions. This result clearly suggests that activation of protein kinase C not only is involved in the contractile response evoked after stimulation of plasma membrane receptors extracellularly, but is also the mechanism leading to contraction activated by arachidonic acid from the intracellular side of the plasma membrane. In accordance with this finding, different protein kinase C isoforms can be activated by arachidonic acid (Lester et al., 1991; Khan et al., 1995); arachidonic acid stimulates protein kinase C binding to actin filaments (Prekeris et al., 1996) and arachidonic acid was reported to sensitize the contractile smooth muscle apparatus to basal Ca^{2+} concentrations (Gong et al., 1992).

A second messenger role for arachidonic acid in signal transduction is widely accepted (Axelrod, 1990; Khan et al., 1995). Apart from physiologically controlled arachidonic acid formation after receptor stimulation, free fatty acids also accumulate in cells under nonphysiological circumstances, e.g., tissue anoxia (Sakaida et al., 1992).

Experimental evidence suggests that changes in intracellular arachidonic acid concentration can account for pathological effects such as ischemia and neurotrauma (Bazan et al., 1995; Oe et al., 1994; Katsura et al., 1993). The presence of arachidonic acid on either side of plasma membrane under such pathological conditions can determine the vascular tone. Furthermore, arachidonic acid stimulates the NADPH/NADH oxidase systems that produce superoxide anions (Griendling et al., 1994). These systems modulate vascular tone (Pagano et al., 1995; Rajagopalan et al., 1996) and are especially involved in angiotensin II-induced hypertension (Griendling et al., 1994; Fukui et al., 1997).

In conclusion, we showed that different mechanisms are involved in the contractile effect of intracellular and in the effect of extracellular arachidonic acid in rat aorta. The characteristics of the contractions induced by extracellular arachidonic acid resemble those of agonist-induced contractile responses. In contrast, contractions induced by arachidonic acid on the intracellular side of the plasma membrane do not involve Ca^{2+} entry via voltage-operated channels and activation of calmodulin. This contraction is only mediated by protein kinase C activation.

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