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Calcium-Calmodulin Plays a Major Role in Bradykinin-Induced Arachidonic Acid Release by Bovine Aortic Endothelial Cells

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Abstract We provided evidence that calcium-calmodulin plays a major role in bradykinin-induced arachidonic acid release by bovine aortic endothelial cells. In cells labeled for 16 hr with ^{3}H-arachidonic acid, ionomycin and Ca^{2+}-mobilizing hormones such as bradykinin, thrombin and platelet activating factor induced arachidonic acid release. However, arachidonic acid release was not induced by agents known to increase cyclic AMP (forskolin, isoproterenol) or cyclic GMP (sodium nitroprusside). Bradykinin induced the release of arachidonic acid in a dose-dependent manner (EC_{50} = 1.6 ± 0.7 nM). This increase was rapid, reaching a maximal value of fourfold above basal level in 15 min. In a Ca^{2+}-free medium, bradykinin was still able to release arachidonic acid but with a lower efficiency. Quinacrine (300 μM), a blocker of PLA_2, completely inhibited bradykinin-induced arachidonic acid release. The B_2 bradykinin receptor antagonist HOE-140 completely inhibited bradykinin-induced arachidonic acid release. The B_1-selective agonist DesArg^9-bradykinin was inactive and the B_1-selective antagonist [Leu^8]DesArg^9-bradykinin had no significant effect on bradykinin-induced arachidonic acid release. The phospholipase C inhibitor U-73122 (100 μM) decreased bradykinin-induced arachidonic acid release. The calmodulin inhibitor W-7 (50 μM) drastically reduced the bradykinin- and ionomycin-induced arachidonic acid release. Also, forskolin decreased bradykinin-induced arachidonic acid release. These results suggest that the activation of PLA_2 by bradykinin in BAEC is a direct consequence of phospholipase C activation. Ca^{2+}-calmodulin appears to be the prominent activator of PLA_2 in this system.

Key words: calmodulin, bradykinin, phospholipase A_2, endothelial cells, arachidonic acid

The endothelium plays an important role in the regulation of vascular tone [Vanhoutte et al., 1986; Henrich, 1991; Himmel et al., 1993]. In response to a variety of external stimuli, endothelial cells secrete potent vasoactive compounds such as endothelin [Yanagisawa et al., 1988], nitric oxide [Moncada et al., 1991], and prostaglandins [Moncada et al., 1977]. The rate-limiting step for the synthesis of prostaglandins and related compounds is the release of arachidonic acid (AA) from membrane phospholipids [Glaser et al., 1993]. Two major pathways of AA release are generally accepted [Smith, 1989]. It can be released directly from phospholipids through the action of phospholipase A_2 (PLA_2) or it can be released from sequential cleavage of phosphatidylinositols by phospholipase C (PLC) and diacylglycerol lipase. Although the mechanisms are not completely understood, bradykinin and other Ca^{2+}-mobilizing agonists appear to promote AA release by elevating intracellular Ca^{2+} concentrations and activating PLA_2 [Lambert et al., 1986]. At least four regulatory mechanisms have been proposed to promote activation of PLA_2: (1) an increase in the concentration of cytosolic free Ca^{2+} may be sufficient to evoke an activation of PLA_2 [Rittenhouse and Horne, 1984]; (2) lipids generated by receptor-mediated stimulation of phosphoinositide metabolism may function to locally perturb the substrate membrane phospholipids, thereby facilitating their hydrolysis by PLA_2 [Sato et al., 1992]; (3) G proteins in many cellular systems may stimulate PLA_2 independently of the PLC and protein kinase C signaling pathways [Axelrod, 1990]; and (4) PLA_2 may be regulated by phosphorylation [Kramer et al., 1993]. Protein kinase C [Halenda et al., 1989], protein kinase A [Wightman et al., 1982], and tyrosine kinases [Gold-

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berg et al., 1990] have been implicated in the direct activation of PLA_2.

Understanding the signal transduction mechanisms that regulate PLA_2 activity and AA release is central to elucidate the physiological processes in which the endothelium is involved (homeostasis, cellular proliferation, inflammation, and immunity). The multiple forms of PLA_2 that can be expressed in a single cell [Glaser et al., 1993] and the possibility of multiple pathways, involving different enzymes acting sometimes in synergy to release AA, are complicating the mechanistic studies. Furthermore, different regulatory mechanisms may be used by different cell types or by cell types from different species. For these reasons, despite the numerous studies that have been conducted so far, the regulation of PLA_2 in endothelial and other cells is incompletely understood. Because bovine aortic endothelial cells (BAEC) represent a very commonly used model to study the endothelium at the cellular level, the aim of the present work was to investigate the mechanism by which Ca^{2+}-mobilizing hormones (more specifically bradykinin) stimulate AA release from BAEC.

MATERIALS AND METHODS

Materials

^{3}H-arachidonic acid (1 mCi/ml) was purchased from Amersham Life Science (Oakville, Ontario). Bradykinin, thrombin, angiotensin II, quinacrine, forskolin, isoproterenol, and sodium nitroprusside were purchased from Sigma (St. Louis, MO). PAF (Platelet Activating Factor), DesArg^9-bradykinin, [Leu^8]DesArg^9-bradykinin, and[D-Arg^0,Hyp^3,B-(2-Thienyl)-Ala^5,D-Tic^7, Oic^8]-bradykinin (HOE 140) were purchased from Bachem (Torrance, CA). Ionomycin, W-7 (N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide, HCl), and fura-2/AM were purchased from Calbiochem (La Jolla, CA). U-73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)-amino)-hexyl)-1H-pyrrole-2,5-dione) was a generous gift from The Upjohn Company (Kalamazoo, MI).

Cell Culture

Bovine thoracic aortas were excised and washed immediately with ice-cold sterile phosphate-buffered saline (pH 7.4: 3.5 mM NaH_2PO_4, 16.5 mM Na_2HPO_4, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 3.5 mM KCl, and 135 mM NaCl). The aortas were cleaned of connective tissue under sterile conditions, opened longitudinally, and fixed on a plate with the intimal face upward. The endothelial surface was carefully scraped and then incubated for 15 min at 37°C with 30 ml (for 4-6 aortas) of M199 medium (pH 7.4) containing: 25 mM HEPES, 27 mM $NaHCO_3$, 100 U/ml of penicillin and 0.1 mg/ml of streptomycin, 1 mg/ml of collagenase, and 2.5 μg/ml of fungizone. After centrifugation at 500 × *g* for 10 min, the pellet was resuspended in 6 ml of cell culture medium M199 supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, 60 μg/ml streptomycin, 2 mM L-glutamine, and $NaHCO_3$ 7.5%. The cells were plated in a cell culture flask (25 cm^2, Falcon) and maintained in a 95% air–5% CO_2 humidified incubator at 37°C. The culture medium was changed the day after seeding. Results presented in these studies were obtained with cells from passages 5–25.

The cells were identified as endothelial cells based on their ability to form a typical cobblestone appearance when confluent and take up fluorescently labeled acetylated low-density lipoprotein (data not shown).

Release of ^{3}H-Arachidonic Acid

BAEC were grown to confluence in 24-multiwell plates. After 2 days of culture, the medium was replaced with 1 ml of DMEM containing 0.5 μCi/ml of ^{3}H-arachidonic acid and cells were labeled for 16 hr. After four washing steps with incubation medium (25 mM HEPES, pH 7.2, 150 mM NaCl, 5 mM KCl, 5.5 mM dextrose, 0.8 mM $MgSO_4$, 1 mM $CaCl_2$, 0.1% BSA) at 37°C, the cells were incubated for 15 min at 37°C in 0.5 ml of medium in the presence or absence of inhibitor. Each inhibitor was used within a range of concentrations at which it is commonly used to inactivate its specific target. Test agents were then added to stimulate the cells for another 15 min (for dose–response curves). Medium was removed, and its radioactive content was determined by scintillation counting.

Determination of Intracellular Ca^{2+}

BAEC were grown to confluence on glass coverslips (13 mm × 22 mm). The coverslips were rinsed with a medium containing 135 mM NaCl, 5.3 mM KCl, 1.2 mM $CaCl_2$, 1 mM $MgSO_4$, 5.5 mM dextrose, and 10 mM HEPES, pH 7.2, and were then incubated for 45 min at 37°C with 3 μM fura-2/AM. After two washing steps with 2 ml of the same medium, coverslips were incubated for 15 min at room temperature to allow

the hydrolysis of the dye, and placed in a cuvette filled with 2 ml of the same medium. Fluorescence was measured at room temperature with an Hitachi F-2000 spectrofluorometer with alternating excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm.

Thin-Layer Chromatography Analysis of Released Arachidonic Acid

A modification of the method of Sato et al. [1992] was used to identify AA. BAEC were labeled with ^{3}H-AA and then stimulated with 100 nM bradykinin as described above. After a 15 min incubation, the medium was collected and extracted by adding a mixture of chloroform/methanol/HCl (200/200/1, v/v/v). The mixture was vortexed vigorously and the phases were separated by centrifugation at 1,000 × *g* for 5 min. The upper aqueous phase was discarded. The lower organic phase was dried under a stream of nitrogen and then dissolved in 50 µl of chloroform. The samples were applied together with standards on thin-layer chromatography plates and developed in a solvent system consisting of petroleum ether/diethyl ether/acetic acid (60/45/1, v/v/v). AA was identified with authentic standard revealed by coloration with iodine vapor. The plates were scraped and the radioactivity was determined by scintillation counting. About 50% of the radioactive content of the medium comigrated with AA. Under our chromatographic conditions, the rest of the radioactivity remained at the baseline together with PGE_2 and 6-keto-$PGF_{1\alpha}$ standards.

Statistical Analysis

All the data are expressed as mean ± SD. The statistical significance ($P < 0.05$ or $P < 0.01$) of the observed ^{3}H-AA release was assessed with Student's *t*-test for unpaired data or with a one-way ANOVA followed by a Dunnet multiple comparisons test.

RESULTS

It has been shown that the activation of PLA_2 and the release of AA in different cell types can be stimulated either by Ca^{2+}-mobilizing agonists [Lambert et al., 1986] or by agents that directly increase intracellular Ca^{2+} concentrations (such as ionomycin) [Rittenhouse and Horne, 1984]. In this study, we evaluated the effect of different Ca^{2+}-mobilizing agonists and other agents on AA release from BAEC. As shown in Figure 1, the release of ^{3}H-AA was

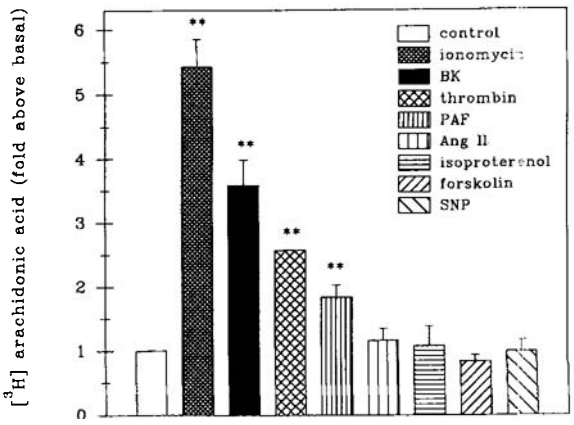

Fig. 1. Effect of different agents on the release of ^{3}H-AA by BAEC. The ^{3}H-AA-labeled cells were stimulated with ionomycin (10 µM), bradykinin (BK, 100 nM), thrombin (1 U/ml), PAF (10 µM), angiotensin II (Ang II, 1 µM), isoproterenol (1 µM), forskolin (1 µM), or sodium nitroprusside (SNP, 100 µM) for 15 min. The radioactivity released into the medium was then determined. Each value represents the mean ± SD of triplicate determinations. ** $P < 0.01$, as compared to the control according to Student's *t*-test. Similar results were obtained in three independent experiments.

stimulated by known Ca^{2+}-mobilizing agents such as ionomycin (5.4-fold), bradykinin (3.6-fold), thrombin (2.6-fold), and PAF (1.8-fold). Unexpectedly, angiotensin II, a well-known Ca^{2+}-mobilizing hormone, had no significant effect. Forskolin and isoproterenol (known to increase cyclic AMP) and sodium nitroprusside (known to increase cyclic GMP) were unable to stimulate AA release from BAEC. Due to its potent action on ^{3}H-AA release, bradykinin was the Ca^{2+}-mobilizing hormone used to further characterize ^{3}H-AA release from BAEC.

Effect of Bradykinin on $[Ca^{2+}]_i$

Since Ca^{2+} seems to play an important role in the release of AA, we verified the effect of bradykinin on intracellular Ca^{2+} concentrations. Stimulation of BAEC with 100 nM bradykinin, in the presence of external Ca^{2+} (1 mM), produced a typical biphasic response composed of an initial spike followed by a sustained phase maintained above the basal level (Fig. 2A). In the absence of extracellular Ca^{2+}, the initial spike was still observed but the sustained phase was abolished. These results suggest that the initial spike of Ca^{2+} produced by bradykinin results from the release of Ca^{2+} from intracellular stores, while the sustained phase depends on the influx of extracellular Ca^{2+}. These findings are consistent with the notion that the bradyki-

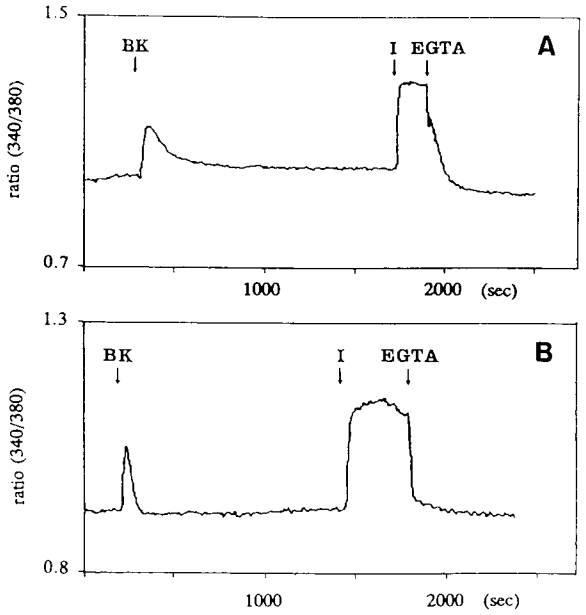

Fig. 2. Effect of bradykinin on $[Ca^{2+}]_i$ in fura-2-loaded BAEC. The cells were stimulated in the presence (**A**) or absence (**B**) of extracellular Ca^{2+} (1 mM). The arrows indicate the time of addition of bradykinin (BK, 100 nM), ionomycin (I, 25 μM), or EGTA (5 mM). Similar results were obtained in three independent experiments.

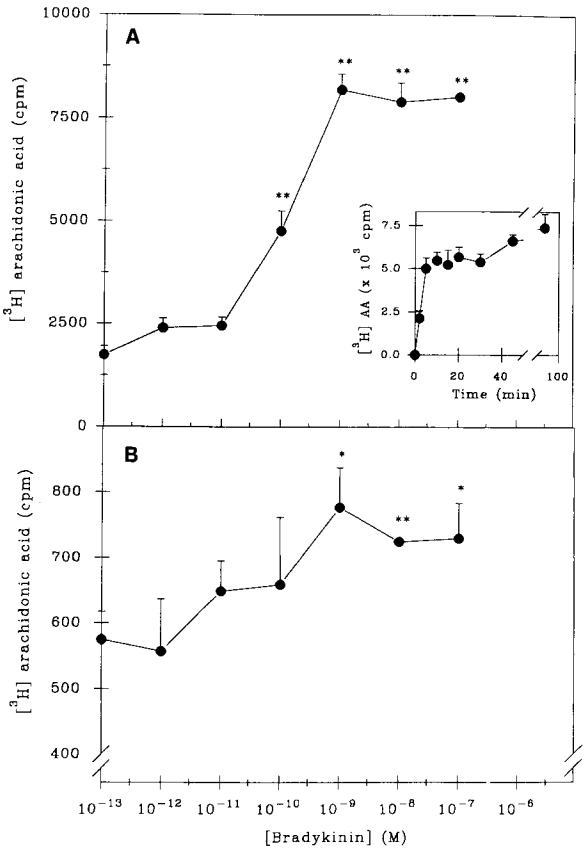

Fig. 3. Dose-dependent effect of bradykinin on the release of ^{3}H-AA by BAEC. The ^{3}H-AA-labeled cells were stimulated for 15 min with the indicated concentrations of bradykinin (BK) in the presence (**A**) or absence (**B**) of 1 mM external Ca^{2+}. Radioactivity released into the medium was then determined. The time course of 100 nM bradykinin effect is shown in the inset. Each value represents the mean ± SD of triplicate determinations. $^*P < 0.05$ and $^{**}P < 0.01$, as compared to the control. In A, the statistical significance was assessed with one-way ANOVA followed by a Dunnet multiple comparisons test. In B, the statistical significance was assessed with Student's *t*-test. For the time course, all values denote significant difference ($P < 0.01$) from control according to Student's *t*-test. Similar results were obtained in three and two independent experiments in A and B, respectively.

nin-elicited transient Ca^{2+} response is dependent both on extracellular Ca^{2+} and intracellular Ca^{2+}, and that the sustained phase is entirely dependent on extracellular Ca^{2+}. Under the same conditions, 1 μM angiotensin II did not produce any significant change in the intracellular Ca^{2+} concentration of BAEC (data not shown).

Effect of Bradykinin on ^{3}H-AA Release

To assess the effect of extracellular Ca^{2+} on the release of AA, BAEC were incubated in a medium with or without $CaCl_2$ and then stimulated with bradykinin. In the presence of 1 mM external Ca^{2+}, bradykinin induced the release of ^{3}H-AA in a dose-dependent manner, reaching a maximal value of more than threefold above basal level at a concentration of 10 nM (from 2,455 cpm to 8,021 cpm), with an EC_{50} of 1.6 ± 0.7 nM (Fig. 3A). ^{3}H-AA release was rapid and half maximal stimulation was observed within 5 min, reaching a plateau at 15 min (Fig. 3A, inset). In order to evaluate the effect of extracellular Ca^{2+} on bradykinin-stimulated ^{3}H-AA release, bradykinin stimulation was performed in a Ca^{2+}-free medium. Under these conditions, bradykinin induced a smaller release of ^{3}H-AA in a dose-dependent manner, reaching a maximal value of 1.3-fold above basal level at a concentration of 10 nM, with an EC_{50} of 0.2 nM (average of two experiments that yielded values of 0.1 nM and 0.3 nM; Fig. 3B). These results suggest that the release of Ca^{2+} from intracellular stores is sufficient to induce some release of AA by BAEC, but also suggest that the extracellular Ca^{2+} is playing an important role in the overall process activated by bradykinin.

Effect of Quinacrine on Bradykinin-Induced ^{3}H-AA Release

AA can be released directly from membrane phospholipids through the action of PLA_2

[Smith, 1989]. To determine whether bradykinin-induced AA release was the result of PLA_2 activation, BAEC were incubated with quinacrine, a potent blocker of PLA_2, 15 min prior to their stimulation with bradykinin. Pretreatment with quinacrine, which by itself had no effect on ^{3}H-AA release (data not shown), completely inhibited bradykinin-induced ^{3}H-AA release. The effect of quinacrine was dose-dependent, with an IC_{50} of 85 ± 7 μM (Fig. 4). These results suggest that the release of AA induced by bradykinin is principally mediated by the action of PLA_2.

Identification of the Receptor-Type Mediating Bradykinin-Induced ^{3}H-AA Release by BAEC

In order to determine the type of receptor involved in bradykinin-stimulated ^{3}H-AA release, we examined the effect of selective bradykinin analogs. Figure 5 shows that 10 μM DesArg^{9}bradykinin, a B_1-selective agonist, did not induce any significant release of AA by BAEC. Consequently, the B_1-selective receptor antagonist [Leu^8]DesArg^{9}bradykinin (10 μM) did not affect the release of AA induced by bradykinin. Pretreatment of BAEC with 10 μM HOE 140, a selective B_2 receptor antagonist, completely inhibited bradykinin-induced ^{3}H-AA release. These results demonstrate that bradykinin stimulates the release of AA from BAEC through a B_2 receptor.

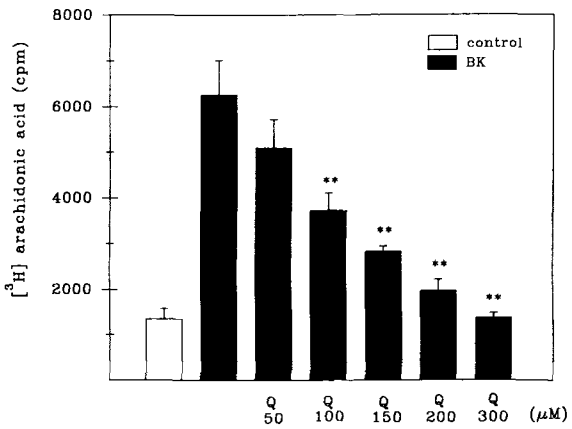

Fig. 4. Effect of quinacrine on the bradykinin-induced ^{3}H-AA release by BAEC. The ^{3}H-AA-labeled cells were pretreated for 15 min with various doses of quinacrine (Q). They were then stimulated with 100 nM bradykinin (BK) for 15 min. Radioactivity released into the medium was then determined. Each value represents the mean ± SD of triplicate determinations. $^{**}P < 0.01$, as compared to the BK stimulation according to one-way ANOVA followed by a Dunnet multiple comparisons test. Similar results were obtained in three independent experiments.

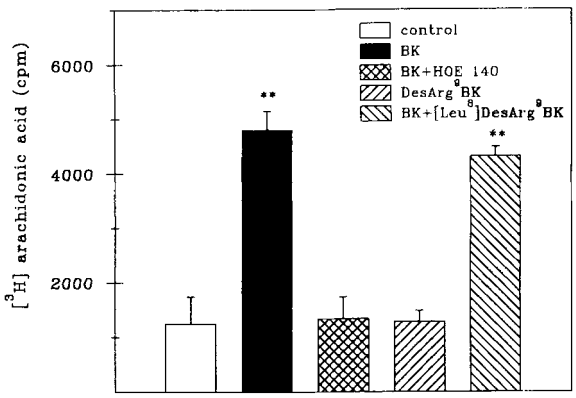

Fig. 5. Effects of bradykinin analogs on the release of ^{3}H-AA from BAEC. The ^{3}H-AA-labeled cells were stimulated with either bradykinin (BK, 100 nM) or DesArg^9 BK (10 μM). The antagonists HOE 140 (10 μM) and [Leu^8]DesArg^{9}BK (10 μM) were added 15 min before stimulation with BK. Radioactivity released into the medium was then determined. Each value represents the mean ± SD of triplicate determinations. $^{**}P < 0.01$, as compared to the control according to Student's *t*-test. Similar results were obtained in three independent experiments.

Effect of PLC Inhibitor on Bradykinin-Induced ^{3}H-AA Release

To determine what role PLC might play in the activation of AA release by bradykinin, we studied the effect of U-73122, a potent PLC inhibitor. Pretreatment with U-73122, which by itself had no effect on the release of AA (data not shown), decreased very substantially the effect of bradykinin. The inhibitory effect of U-73122 was dose dependent, with an IC_{50} of 13.8 ± 5.1 μM (Fig. 6). These results are consistent with the idea that PLC is an important step in the activation of PLA_2 by bradykinin in BAEC.

Effect of Calmodulin Inhibitor on Bradykinin- and Ionomycin-Induced ^{3}H-AA Release

Since it is known that bradykinin acts through a Ca^{2+}-dependent mechanism and many calcium-mediated events require the formation of Ca^{2+}-calmodulin complex, we sought to determine whether calmodulin was involved in the activation of PLA_2 by bradykinin in BAEC. W-7 is a known Ca^{2+}-calmodulin inhibitor which binds to calmodulin, prevents its action, and inhibits Ca^{2+}-calmodulin-regulated enzyme activities [Hidaka et al., 1981]. W-7, which by itself had no effect on AA release (data not shown), drastically reduced bradykinin-induced ^{3}H-AA release (Fig. 7A). The effect of W-7 was dose dependent, with an IC_{50} of 10.1 ± 3.6 μM. Similarly, W-7 completely inhibited ionomycin-induced ^{3}H-AA release, with an IC_{50} of 10.4 ± 3.9 μM (Fig. 7B).

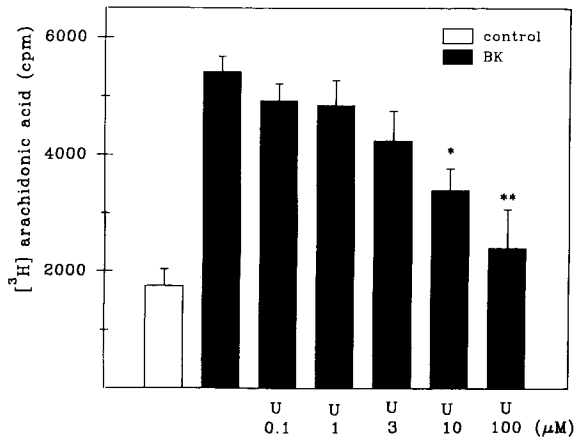

Fig. 6. Effect of U-73122 on the bradykinin-induced ^{3}H-AA release by BAEC. The ^{3}H-AA-labeled cells were pretreated for 15 min with various doses of U-73122 (U) prior to stimulation with 100 nM bradykinin (BK). Radioactivity released into the medium was then determined. Each value represents the mean ± SD of triplicate determinations. $*P < 0.05$ and $**P < 0.01$, as compared to the BK stimulation according to one-way ANOVA followed by a Dunnet multiple comparisons test. Similar results were obtained in three independent experiments.

These results suggest that Ca^{2+}-calmodulin plays an essential role in bradykinin-induced activation of PLA_2.

Cyclic AMP Regulation of Bradykinin-Induced ^{3}H-AA Release

Because in many cellular systems cyclic AMP and Ca^{2+} are known biochemical antagonists, we also investigated the influence of cyclic AMP on the release of AA by BAEC. Figure 8 shows that forskolin, a known activator of adenylyl cyclase, strongly inhibited bradykinin-induced AA release by BAEC. The effect of forskolin was dose dependent, with an IC_{50} of 2.5 ± 1.1 μM.

DISCUSSION

It has been shown that PLA_2 can be activated by physiological relevant increases of intracellular Ca^{2+} [Rittenhouse and Horne, 1984]. This activation of PLA_2 results in the translocation of PLA_2 from the cytosol to the plasma membrane, where phospholipid substrates are localized [Clark et al., 1991]. In endothelial cells, the release of AA could be triggered by Ca^{2+}-mobilizing hormones or by agents that directly release Ca^{2+} from intracellular stores [Lambert et al., 1986; Rittenhouse and Horne, 1984]. The aim of this work was to study the steps leading to the release of AA during the stimulation of BAEC with Ca^{2+}-mobilizing hormones.

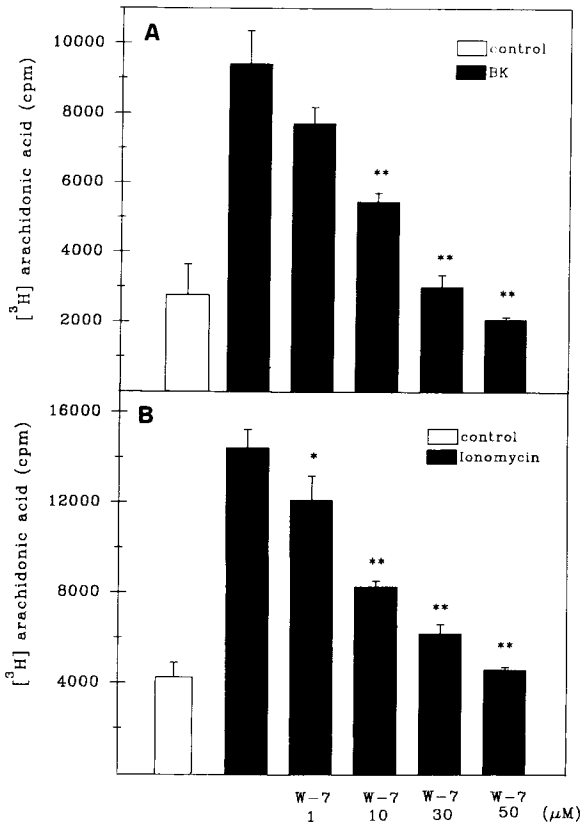

Fig. 7. Effect of W-7 on bradykinin- (**A**) and ionomycin-induced (**B**) ^{3}H-AA release by BAEC. The ^{3}H-AA-labeled cells were pretreated for 15 min with various doses of W-7, prior to stimulation with 100 nM bradykinin (BK) or 10 μM ionomycin for 15 min. Radioactivity released into the medium was then determined. Each value represents the mean ± SD of triplicate determinations. $*P < 0.05$ and $**P < 0.01$, as compared to the BK stimulation according to one-way ANOVA followed by a Dunnet multiple comparisons test. Similar results were obtained in three independent experiments.

We have shown that bradykinin, thrombin, PAF, and ionomycin increased the release of AA by BAEC, whereas forskolin, isoproterenol, and sodium nitroprusside did not increase this activity. These results indicate that the release of AA by BAEC is activated exclusively by Ca^{2+}-mobilizing agents. This is in agreement with previous studies showing that bradykinin, ATP, thrombin, and histamine increase the release of AA from different types of endothelial cells [Whorton et al., 1984; Hallam and Pearson, 1986; Hong et al., 1985; Tokumoto et al., 1994]. Interestingly, angiotensin II, a known Ca^{2+}-mobilizing hormone, did not affect the release of AA by BAEC. Whorton et al. [1982] also reported that angiotensin II has no effect on the release of AA by porcine aortic endothelial cells. Our results also showed that angiotensin II does not pro-

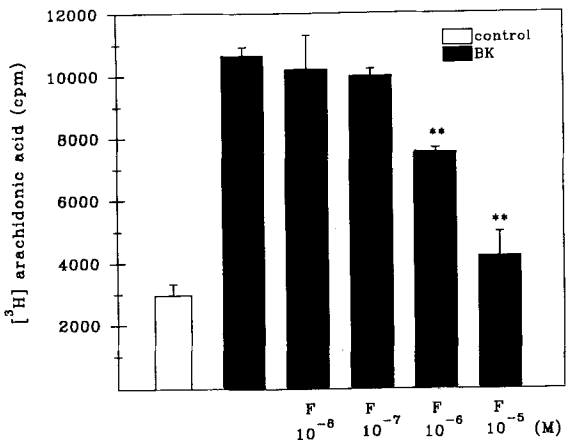

Fig. 8. Effect of forskolin on bradykinin-induced ^{3}H-AA release by BAEC. The ^{3}H-AA-labeled cells were pretreated for 15 min with various doses of forskolin (F), prior to stimulation with 100 nM bradykinin (BK) for 15 min. Radioactivity released into the medium was then determined. Each value represents the mean ± SD of triplicate determinations. $^{**}P < 0.01$, as compared to the BK stimulation according to one-way ANOVA followed by a Dunnet multiple comparisons test. Similar results were obtained in three independent experiments.

duce any significant increase of Ca^{2+} in fura-2–loaded BAEC. These results suggest that BAEC do not express functional angiotensin II receptor. Although a fragment of angiotensin II is interacting with a specific receptor on BAEC, this receptor is different from the classical AT_1 or AT_2 receptor, and does not promote Ca^{2+} mobilization [Bernier et al., 1995].

The biphasic Ca^{2+} response of BAEC stimulated with bradykinin consists of a sharp rise of intracellular Ca^{2+} concentration followed by a lower sustained plateau phase. This classical biphasic response is similar to that of most cells in response to Ca^{2+}-mobilizing hormones [Putney, 1987; Buchan and Martin, 1991; Ricupero et al., 1993]. The initial transient elevation of $[Ca^{2+}]_i$ depends on polyphosphoinositide hydrolysis, resulting in the synthesis of IP_3, which then causes the release of Ca^{2+} from endoplasmic reticulum [Berridge and Irvine, 1984]. The sustained plateau phase depends on Ca^{2+} influx [Hallam et al., 1988]. The ability of bradykinin to cause a transient elevation of Ca^{2+} in the absence of extracellular Ca^{2+} and the absence of a sustained plateau phase under these conditions are consistent with such an interpretation. These results indicate that bradykinin is activating a classical mechanism of Ca^{2+} mobilization *in* BAEC.

We showed that bradykinin caused a dose- and time-dependent AA release by BAEC. The release was rapid, reaching a maximal value of about threefold above basal level within 15 min. In a Ca^{2+}-free medium, bradykinin was still able to release AA but with a much lower efficiency. Brooks et al. [1989] have shown in rat glioma cells activated with acetylcholine that AA release is completely dependent on the influx of extracellular Ca^{2+}. Our results indicate that the external Ca^{2+} is not essential for AA release by BAEC but it seems that the maintenance of an elevated level of Ca^{2+} is required to obtain an optimal release. This is in agreement with the work of Buckley et al. [1991] showing a reduced AA release by porcine aortic endothelial cells stimulated with bradykinin in the absence of extracellular Ca^{2+}. The discrepancy between our results and those of Brooks et al. could be attributed to different mechanisms utilized by BAEC and glioma cells to release AA.

We observed that the release of AA mediated by bradykinin could be completely inhibited in the presence of quinacrine, a specific blocker of PLA_2 [Blackwell and Flower, 1983; Neve et al., 1995]. These results support the view that in BAEC, Ca^{2+}-mobilizing hormones release AA principally through the direct hydrolysis of phospholipids by PLA_2. This was also the mechanism by which bradykinin released AA in porcine aortic endothelial cells [Kaya et al., 1989]. Although the pathway through diacylglycerol lipase seems to contribute importantly to the generation of AA in different cell systems such as murine liver cells activated with erythropoietin [Mason-Garcia et al., 1992] and chromaffin cells activated with acetylcholine [Rindlisbacher et al., 1990], it does not appear to play an important role in bradykinin-induced AA release by BAEC, which was only slightly inhibited in the presence of 10 μM RHC-80267, a specific inhibitor of DAG lipase (data not shown).

Bradykinin-induced AA release from BAEC was completely inhibited by HOE 140, a B_2 receptor antagonist, and was not affected by [Leu^8]DesArg^{9}bradykinin, a B_1 receptor antagonist. DesArg^{9}bradykinin, a B_1 receptor agonist, did not induce AA release. Based on the classification suggested by Regoli and Barabe [1980], the bradykinin receptor involved in bradykinin-induced AA release is a B_2 receptor. Previous studies also reported the presence of this receptor type on bovine endothelial cells [Morgan-Boyd et al., 1987; Derian and Moskowitz, 1986]. Bradykinin B_2 receptor is coupled to PLC in endothelial cells and thus activates the hydroly-

sis of PIP_2 and the production of the intracellular mediators IP_3 and diacylglycerol [Lambert et al., 1986]. IP_3 activates a specific receptor on the endoplasmic reticulum, causing the release of stored Ca^{2+} [Berridge and Irvine, 1984], whereas diacylglycerol activates protein kinase C. We have shown that the PLC inhibitor U-73122 [Bleasdale et al., 1990; Jin et al., 1994] considerably diminished bradykinin-induced AA release. Bradykinin-induced AA release thus appears to be secondary to the activation of PLC. This conclusion was also reached by Lambert et al. [1986], Derian and Moskowitz [1986], and Jaffe et al. [1987], who suggested that PLA_2 activation was the consequence of PIP_2-hydrolyzing PLC activation. Therefore the inhibitory effect of U-73122 results from a blunting of either the Ca^{2+} signal or protein kinase C activation by diacylglycerol. The complete inhibition of bradykinin-induced AA release by the calmodulin inhibitor W-7 strongly suggests that Ca^{2+} is the principal second messenger involved in the process. W-7 also abolished the potent effect of ionomycin, demonstrating that in BAEC, Ca^{2+}-calmodulin is the prominent activator of PLA_2. In porcine aortic endothelial cells, Whorton et al. [1984] also reported that AA release is dependent on the activation of a Ca^{2+}-dependent phospholipase by a calmodulin-mediated mechanism. In Madin-Darby canine kidney cells [Weiss and Insel, 1991] and in platelets [Halenda et al., 1989], protein kinase C has been shown to contribute importantly to the activation of PLA_2. More recently, Lin et al. [1993] have demonstrated that mitogen-activated protein kinase mediates, in part, the agonist-induced activation of PLA_2 in COS-7 cells. Taken together, these studies clearly indicate that, depending on the cell type, there are numerous different pathways that can be used to activate PLA_2. Given the multiple roles of eicosanoids in health and disease, it becomes important to identify in all the different tissues the most active pathway involved in AA release. In BAEC, it appears that Ca^{2+}-calmodulin is the prominent activator of PLA_2. Since a similar conclusion was drawn from studies with porcine aortic endothelial cells, it is tempting to suggest that most endothelial cells are preferentially using this pathway. Obviously these studies need to be repeated with endothelial cells from different vascular beds in order to reach that conclusion.

Interestingly, we have shown that forskolin, a direct activator of adenylyl cyclase, attenuated bradykinin-induced AA release. Similar results were also recently obtained by Kennedy et al. [1995], who showed that forskolin inhibits the effect of bradykinin on AA release by Madin-Darby canine kidney cells. These results suggest that cyclic AMP–dependent processes may be antagonistic to Ca^{2+}-dependent processes in the activation of PLA_2. This is consistent with the report of Lücknoff et al. [1990] that cyclic AMP attenuates the increase of intracellular Ca^{2+} in cells by inhibiting the catalytic activity of phospholipase C. PGI_2, the main AA metabolite liberated from BAEC, is known to interact with a receptor positively coupled to adenylyl cyclase. It is interesting to propose that PGI_2 could activate a negative feedback mechanism to diminish its own production by negatively regulating PLA_2 activity.

In conclusion, we have shown that the activation of PLA_2 by bradykinin in BAEC is a direct consequence of phospholipase C activation. Ca^{2+}-calmodulin appears to be the prominent activator of PLA_2 in this system. The stimulatory effect of the Ca^{2+} pathway on PLA_2 activation is antagonized by the cyclic AMP pathway. Further work is necessary to identify the specific molecular components involved in these processes and to clarify their precise role in the regulation of PLA_2 activity. However, it is important to consider that in BAEC, any attempt to increase intracellular Ca^{2+} or to activate PLC will cause secondary activation of PLA_2 and release of AA metabolites.

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