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Regulation of arachidonic acid release in vascular endothelium

Ca^{2+}-dependent and -independent pathways

Barbara J. BUCKLEY*‡, Aaron BARCHOWSKY,†§ Rowena J. DOLOR† and A. Richard WHORTON†
Departments of *Medicine and †Pharmacology, Duke University Medical Center, Durham, NC 27710, U.S.A.

Ca^{2+} metabolism and its relationship to arachidonic acid release were studied in cultured pig aortic endothelial cells. When cells were treated with bradykinin, a rapid rise in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) occurred. Arachidonic acid release from cells prelabelled with [^{3}H]arachidonic acid and subjected to flow-through conditions closely followed the changes in $[Ca^{2+}]_i$. Attenuation of the Ca^{2+} response by chelating extracellular and intracellular Ca^{2+} or by desensitization of receptors led to comparable attenuation of arachidonate release. Activation of protein kinase C inhibited Ca^{2+} mobilization in response to bradykinin and stimulated arachidonic acid release. Inhibition of protein kinase C had no effect on bradykinin-stimulated arachidonic acid release, suggesting that protein kinase C does not mediate the bradykinin response. The role of GTP-binding regulatory proteins (G-proteins) in mediating the bradykinin response was also investigated. Bradykinin-stimulated arachidonic acid release was not diminished by preincubation with pertussis toxin. Treatment with the G-protein activator AlF_4^- resulted in the release of a large pool of arachidonic acid and the formation of lysophospholipids. Combined treatment with AlF_4^- and bradykinin resulted in a greater than additive effect on arachidonic acid release. In contrast with bradykinin, AlF_4^--stimulated arachidonic acid release was not dependent on the presence of extracellular Ca^{2+} or the mobilization of intracellular Ca^{2+}. These results demonstrate Ca^{2+}-dependent (bradykinin) and Ca^{2+}-independent (AlF_4^-) pathways of phospholipase A_2 activation.

INTRODUCTION

Prostacyclin production by endothelial cells mediates several important vascular functions, including regulation of platelet aggregation, regulation of neutrophil adherence and regulation of vascular tone [1,2]. Prostaglandin synthesis by these and other cells is dependent on the availability of unesterified arachidonic acid, the substrate for prostaglandin H synthase. Although the mechanisms are not completely understood, agonists such as bradykinin, thrombin and ATP appear to promote arachidonic acid release from phospholipids by elevating intracellular Ca^{2+} concentrations ($[Ca^{2+}]_i$) [3,4] and activating phospholipase A_2 [5,6]. It has been shown that bradykinin activates phospholipase C in endothelial cells, leading to the hydrolysis of PtdIns(4,5)P_2 and the production of the intracellular mediators Ins(1,4,5)P_3 and diacylglycerol [5,6]. Ins(1,4,5)P_3 binds to specific receptors on the endoplasmic reticulum, causing the release of stored Ca^{2+} [7], whereas diacylglycerol activates protein kinase C, which down-regulates receptor function [8] and activates certain genes [9].

In our laboratory, kinetic studies of the bradykinin response in endothelial cells have shown that Ins(1,4,5)P_3 production increases rapidly to a peak at 30 s and returns to baseline by 60 s [5,10]. The influx of Ca^{2+} occurs equally as rapidly [3,4] as does the appearance of lysophosphatidylinositol [5,6]. However, unlike Ins(1,4,5)P_3, Ca^{2+} mobilization and lysophospholipid formation continue for 4–6 min before reaching a plateau. The release of arachidonic acid is stimulated immediately after bradykinin administration, reaching a peak at 2–4 min and returning to baseline by 10 min [3]. Hence, the temporal relationship between Ins(1,4,5)P_3 production, Ca^{2+} influx, lysophosphatidylinositol production and arachidonic acid release supports the proposal that Ins(1,4,5)P_3-mediated Ca^{2+} release and the subsequent influx of Ca^{2+} stimulate phospholipase A_2. However, the kinetic and quantitative relationships between Ca^{2+} concentration and arachidonate release have not been investigated.

Furthermore, Ca^{2+}-independent pathways of arachidonic acid release have been proposed. A recent study demonstrated phospholipase A_2 activation in the absence of Ins(1,4,5)P_3 production in bradykinin-stimulated endothelial cells exposed to low temperatures [11]. These findings suggest that phospholipase A_2 may be activated independently of a phospholipase C-mediated increase in $[Ca^{2+}]_i$. In addition, phospholipase A_2 can be activated by a protein kinase C-dependent mechanism [12,13]. This effect does not depend on the influx of extracellular Ca^{2+} [12] or the mobilization of intracellular Ca^{2+} [13], and may be due to enhanced sensivity of phospholipase A_2 to Ca^{2+} [13]. Furthermore, non-hydrolysable GTP analogues stimulate phospholipase A_2 in permeabilized cell systems under conditions of low Ca^{2+} [14–17]. Thus it appears that GTP-binding regulatory proteins (G-proteins) are directly coupled to phospholipase A_2. Whether these apparently Ca^{2+}-independent mechanisms play a role in bradykinin-mediated arachidonic acid release in the pig aortic endothelial cell has not been investigated. However, we have recently demonstrated G-protein-mediated activation of phospholipase A_2 in our model [18].

The purpose of the present study was to investigate the Ca^{2+}-dependence of arachidonic acid release in cultured pig aortic endothelial cells. First, we examined the kinetic and quantitative relationship between cytosolic Ca^{2+} levels and arachidonic acid release after stimulation of endothelial cells with bradykinin. Secondly, we investigated the role of protein kinase C in mediating the responses to bradykinin. Thirdly, we determined the Ca^{2+}-dependence of arachidonic acid release after G-protein activation. Our results demonstrate separate pathways of Ca^{2+}-dependent and Ca^{2+}-independent phospholipase A_2 activation in intact endothelial cells.

Abbreviations used: $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; DMEM, Dulbecco's modified Eagle medium; HBSS, Hanks' balanced salt solution; MAPTAM, bis-(2-amino-5-methylphenoxy)ethane-*NNN'N'*-tetra-acetic acid tetra-acetoxymethyl ester; PMA, phorbol 12-myristate 13-acetate.

‡ To whom correspondence should be addressed.
§ Present address: Department of Pharmacology and Toxicology, Dartmouth Medical College, Hanover, NH 03756, U.S.A.

EXPERIMENTAL

Materials

Bradykinin triacetate, arachidonic acid, NaF, collagenase type 1, Pharmacia Cytodex 1 beads and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.); fura-2 AM (acetoxymethyl ester) was from Molecular Probes (Eugene, OR, U.S.A.); MAPTAM [bis-(2-amino-5-methylphenoxy)ethane-*NNN'N'*-tetra-acetic acid tetra-acetoxymethyl ester] and staurosporine were from Calbiochem (San Diego, CA, U.S.A.); fluorescein isothiocyanate-labelled anti-human factor VIII-related antibody was from Atlantic Antibodies (Scarborough, ME, U.S.A.); acetylated low-density lipoprotein labelled with 1,1'-dioctadecyl-1-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate (Di-I-acLDL) was from Biomedical Technologies (Straghton, MA, U.S.A.); pertussis toxin was from List Biological Co. (Campbell, CA, U.S.A.); Dulbecco's modified Eagle medium (DMEM), medium 199, Hanks' balanced salt solution (HBSS), antibiotic/antimycotic (10000 units of penicillin, 10000 units of streptomycin and 25 μg of fungizone/ml) and trypsin/EDTA were from Grand Island Biological Co. (Grand Island, NY, U.S.A.); fetal-calf serum was from Hyclone (Logan, UT, U.S.A.); tissue-culture plasticware was from Nunc (Scientific Technologies, Raleigh, NC, U.S.A.); t.l.c. plates (HP-K) were from Whatman; and radioisotopes were from New England Nuclear (Wilmington, DE, U.S.A.). All solvents used were of h.p.l.c. grade and were obtained from Fisher.

Cell culture

Endothelial cells were isolated and cultured by established methods as previously described [3,5]. Briefly, pig aortae were enzymically digested with collagenase, and isolates were plated on 1.25 cm × 3 cm glass coverslips or tissue-culture plasticware. Cells were incubated at 37 °C in an atmosphere of 10% CO_2 in air in DMEM containing 10% (v/v) fetal-calf serum and 1% antibiotic/antimycotic. Cells cultured on plastic were passaged by treatment with trypsin/EDTA. Endothelial cells exhibited typical cobblestone appearance and were shown to express factor VIII-related antigen and to incorporate Di-I-acLDL specifically as previously reported [3,5,19]. Primary cells and cells in passages 1–3 were used in these studies.

To establish endothelial cell cultures on Cytodex 1 beads, cells isolated from three pig aortae were inoculated into a stir flask containing 50 ml of sterile Cytodex beads, which were equilibrated in culture medium and diluted to a final concentration of 3 mg/ml. To enhance attachment, the beads plus cells were stirred intermittently at 30 rev./min with a 3 min mixing period, followed by a 15 min rest period. After 3 h, the beads were allowed to settle and half of the medium was replaced with fresh medium. Beads were then stirred continuously at 30–35 rev./min. Half of the medium was replaced every 2–3 days until the cells became confluent on the beads. Cell number was increased by adding fresh beads and adjusting the volume of medium accordingly. Alternatively, cultures of cells on beads were initiated by placing equilibrated beads into a flask containing a confluent monolayer of a primary culture of endothelial cells. As the cells migrated on to the beads and became confluent, the beads were removed and used to inoculate fresh beads in a stir flask.

Measurement of $[Ca^{2+}]_i$

To monitor changes in $[Ca^{2+}]_i$, confluent monolayers of cells on glass coverslips were incubated for 1 h at 37 °C with 20 μM-fura-2 ester in HBSS plus 10 mM-Hepes and 0.01% BSA, pH 7.4, as we have previously described [20]. Briefly, fura-2-loaded cells were placed across the diagonal in a 3 ml cuvette fitted with inlet and outlet lines. The cuvette was perfused at 3 ml/min with HBSS containing 10 mM-Hepes and 0.25% BSA, pH 7.4, and maintained at 37 °C. Agonists were added at the final concentration by stopping flow and rapidly exchanging the contents of the cuvette with agonist-containing buffer by using a 20 ml syringe. Flow was re-established and the ratio of fluorescence at emission wavelength 510 nm of cells alternately excited at 350 nm and 385 nm was continuously monitored (5 s/cycle) and used to calculate $[Ca^{2+}]_i$ by published methods [21].

Fatty acid release

To measure arachidonic acid release from endothelial cells on beads, confluent monolayers of endothelial cells were labelled by overnight incubation with 20 μCi of [^{3}H]arachidonate per 2×10^7 cells. The labelled arachidonic acid was dissolved in 100 μl of ethanol and added to beads suspended in 100 ml of DMEM containing 10% fetal-calf serum. After labelling, the beads were loaded into columns (1.0 cm diameter, 1.5 cm height, 1.0 ml total volume; 2×10^6 cells/column) and perfused at 1 ml/min with HBSS plus 10 mM-Hepes containing 0.25% BSA (fatty-acid-free), pH 7.4. The columns were maintained at 37 °C. Unincorporated label was removed by washing for 20 min. The experiment was begun and 1 ml fractions were collected each 1 min and analysed for radioactivity.

To measure the release of arachidonic acid or stearic acid from confluent monolayers of endothelial cells in flasks, cells were labelled by overnight incubation with 0.4 μCi of [^{3}H]arachidonic acid or [^{14}C]stearic acid/ml in DMEM containing 1% fetal-calf serum and 1% antibiotic/antimycotic. Unincorporated label was removed by washing four times with HBSS plus 10 mM-Hepes, pH 7.4. The release of labelled fatty acid into HBSS plus 10 mM-Hepes and 0.25% fatty-acid-free BSA was measured by liquid-scintillation spectroscopy. Prostaglandin products were measured by g.l.c./m.s. analysis of cell medium after addition of deuterated internal standards and derivative formation of samples as we previously described [22]. The major prostaglandin product, prostacyclin, was measured as its stable breakdown product 6-oxo-prostaglandin $F_{1\alpha}$.

Protein kinase C activation

This was determined by measuring the translocation of phospholipid-dependent activity from the cytosol to the membrane fraction. Activity was measured by the extent of phosphorylation of histone H1 with [^{32}P]ATP, as previously described [23].

Lysophospholipid formation

To measure this, confluent monolayers of endothelial cells in tissue-culture flasks were incubated overnight with 1 μCi of [^{3}H]choline/ml or 0.2 μCi of [^{14}C]ethanolamine/ml or for 48 h with 3 μCi of [^{3}H]inositol/ml in media containing 5% fetal-calf serum. After washing of cells four times with HBSS plus 10 mM-Hepes, cells were treated with AlF_4^-. For determination of lysophosphatidylcholine [24] and lysophosphatidylethanolamine [25], the reaction was terminated by addition of 1 ml of ice-cold 70% (v/v) methanol and cells were removed from the flasks by scraping and washing twice with 0.65 ml of 70% methanol. After addition of lysophospholipid as carrier, cell lipids were extracted with 4 ml of chloroform and 0.3 ml of water. The resulting aqueous layer was further extracted with 2 ml of chloroform. Lysophosphatidylcholine ($R_F = 0.13$) and lysophosphatidylethanolamine ($R_F = 0.43$) were separated by t.l.c. in solvent systems of chloroform/methanol/water (13:7:1, by vol.) and chloroform/methanol/triethylamine/water (30:35:34:8, by vol.) respectively. For determination of lysophosphatidylinositol [26], the reaction was terminated by addition of 1 ml of ice-cold

methanol/HCl (100:1, v/v) and flasks were scraped and washed with an additional 1 ml of methanol. After addition of lysophospholipid as carrier, cell lipids were extracted with 4 ml of chloroform and 1 ml of water. The resulting aqueous layer was further extracted with 4 ml of chloroform. Lysophosphatidylinositol ($R_F = 0.34$) was separated on oxalate-impregnated t.l.c. plates in a solvent system of chloroform/methanol/4 M-NH_3 (9:7:2, by vol). After detection by autoradiography, spots corresponding to lysophospholipids were scraped into scintillation vials containing 0.5 ml of water before addition of fluor and radioactivity counting by liquid-scintillation spectroscopy.

Cytotoxicity

Cytotoxicity was determined by quantifying the amount of lactate dehydrogenase released by cells during incubation in HBSS in the presence and absence of AlF_4^-. The amount of lactate dehydrogenase released was compared with the amount contained in cell lysates prepared by solubilizing and sonication in HBSS containing 0.05% Triton X-100. Lactate dehydrogenase was determined by spectrophotometric assay [27].

Statistics

Data were analysed by analysis of variance using PC SAS (SAS Institute, Cary, NC, U.S.A.). Multiple comparisons were made by the methods of Dunnett and Duncan [28].

RESULTS

Bradykinin is a potent agonist of arachidonic acid release and prostacyclin synthesis in endothelial cells. In this study, we wished to determine the Ca^{2+} requirement of this response. Initial experiments correlated the time-dependent changes in $[Ca^{2+}]_i$ with arachidonic acid release. When endothelial cells were stimulated with bradykinin (1 μM), $[Ca^{2+}]_i$ rose rapidly and reached a peak by 30–50 s (Fig. 1*a*). Although bradykinin was present in the cuvette for approx. 2 min, $[Ca^{2+}]_i$ did not return to baseline until 10–12 min. A parallel experiment was carried out in cells which were grown on beads, packed in columns and subjected to flow-through conditions. This experimental protocol allowed us to measure the time-dependent release of label from cells which were prelabelled with [^{3}H]arachidonic acid under conditions in which re-incorporation of labelled fatty acid into cells was minimal. When bradykinin was included in the perfusate, endothelial cells rapidly released label into the buffer (Fig. 1*b*). This response rose more slowly than the Ca^{2+} response, reaching a peak at 2 min and returning to baseline by 12–14 min. Further experiments investigated the requirement of bradykinin-stimulated arachidonic acid release for Ca^{2+} by determining the effects of a diminished Ca^{2+} response on arachidonic acid release. In these experiments, Ca^{2+} mobilization was attenuated by chelation of extracellular Ca^{2+}, by chelation of intracellular Ca^{2+} and by receptor desensitization.

In the first set of experiments, normal buffer Ca^{2+} levels were decreased from 1.4 mM to 0 by omitting Ca^{2+} and adding 1 mM-EGTA. As a result, Ca^{2+} mobilization in bradykinin-stimulated cells was diminished (Fig. 1*a*). Peak $[Ca^{2+}]_i$ values were decreased by 48.6%, and the total Ca^{2+} transient (area under the curve) was decreased by 81.8%. The remaining response represented that portion of Ca^{2+} mobilization contributed by intracellular stores of Ca^{2+}. Similarly, arachidonic acid release was attenuated. Peak values were decreased by 36% and total release (area under the curve) was decreased by 54%. These experiments with extracellular Ca^{2+} chelation demonstrated that a full arachidonic acid-release response required the influx of extracellular Ca^{2+}. However, these studies suggest that intracellular Ca^{2+} mobilization was sufficient for some arachidonic acid release from endothelial cells. These results are in contrast with our previous findings, which failed to demonstrate arachidonic acid release in Ca^{2+}-free buffer containing 1 mM-EGTA [3]. The former studies were carried out under static conditions using endothelial cells grown in tissue culture flasks. Under these conditions, re-incorporation of released arachidonic acid into cellular phospholipids was likely to have resulted in an apparently smaller arachidonic acid release response.

An intracellular Ca^{2+} chelator, MAPTAM [29], was used to

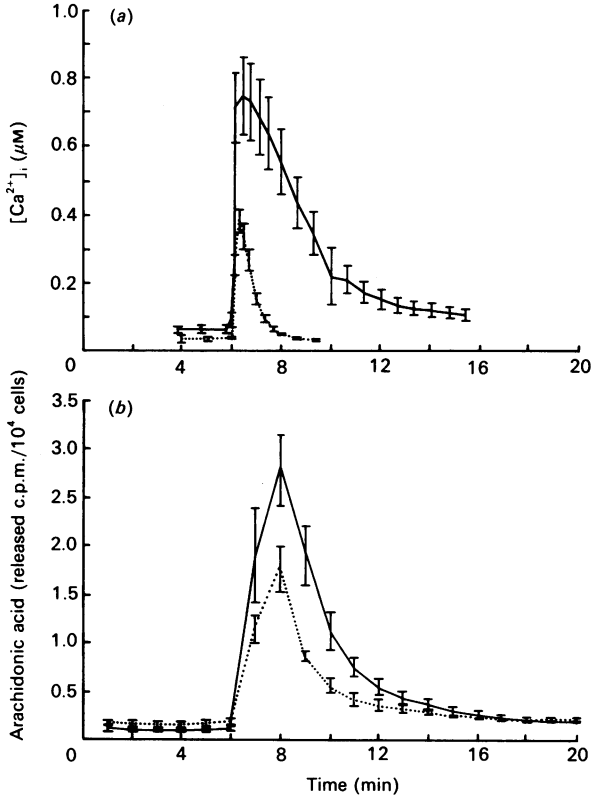

Fig. 1. Effects of bradykinin (*a*) on $[Ca^{2+}]_i$ and (*b*) on arachidonic acid release from endothelial cells

(*a*) Cells were loaded with fura-2 AM as described in the Experimental section. They were placed in the spectrofluorimeter and superfused either with HBSS containing 10 mM-Hepes and 0.25% BSA, pH 7.4, or with Ca^{2+}-free HBSS containing 1 mM-EGTA, 10 mM-Hepes and 0.25% BSA, pH 7.4. At 6 min, 20 ml of either buffer containing 1 μM-bradykinin was rapidly pushed through the cuvette (20 ml/min) under stop-flow conditions. After an additional 1 min, the superfusion was re-started and relative fluorescence was followed as the response returned towards baseline. Data are given as mean $[Ca^{2+}]_i \pm$S.E.M. ($n = 4$). Peak heights and areas were significantly different between the two treatments ($P < 0.01$). (*b*) Endothelial cells were grown on Cytodex 1 beads until confluent. They were labelled by incubation overnight with [^{3}H]arachidonic acid, rinsed and loaded into a glass column as described in the Experimental section. The column was perfused from bottom to top at 1 ml/min with HBSS containing 10 mM-Hepes and 0.25% fatty-acid-free BSA, pH 7.4, and label release was monitored. After a stable baseline was established (5–10 min), 1 min fractions were collected and analysed for label released. Bradykinin was added to the perfusion at 6 min and bradykinin-free buffer was begun at 10 min. Similar experiments were carried out with cells on beads perfused with Ca^{2+}-free buffer containing 1 mM-EGTA. Data are given as means $\pm$S.E.M. ($n = 4$). Peak heights and areas are significantly different between the two treatments ($P < 0.01$). Key: ——, control; ·····, +EGTA.

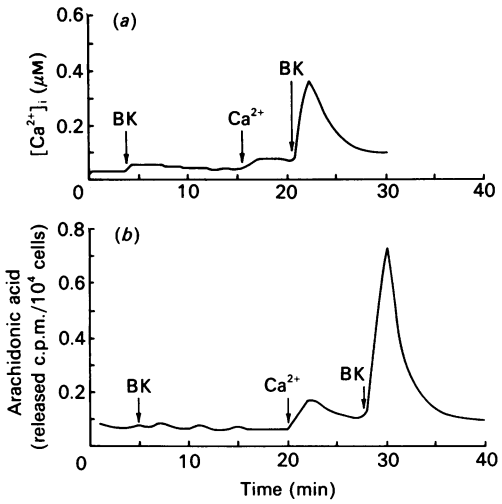

Fig. 2. Effect of MAPTAM (*a*) on $[Ca^{2+}]_i$ and (*b*) on arachidonic acid release from endothelial cells

(*a*) Cells were loaded with fura-2 AM as described in the Experimental section. They were rinsed and incubated for 15 min with 250 μM-MAPTAM-AM in Ca^{2+}-free HBSS containing 1 mM-EGTA, 10 mM-Hepes and 0.25% BSA, pH 7.4. The cells were placed in the spectrofluorimeter and superfused with Ca^{2+}-free buffer. Bradykinin (BK) (1 μM) was given as in Fig. 1(*a*). At 15 min, the superfusion buffer was changed to Ca^{2+}-complete (Ca^{2+}) and bradykinin was given again at 20 min. Data are representative of three similar experiments. (*b*) Cells were labelled with [^{3}H]arachidonic acid as described in the Experimental section. The cells on beads were then incubated for 15 min with 250 μM-MAPTAM-AM in Ca^{2+}-free buffer, rinsed and placed in a column. The column was perfused initially with Ca^{2+}-free buffer and treated after 5 min with bradykinin (BK) (1 μM). After 20 min, the perfusion buffer was changed to Ca^{2+}-complete and a second dose of bradykinin was given at 27 min. Data are representative of three similar experiments.

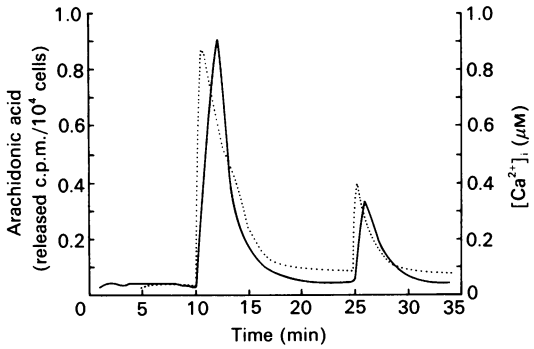

Fig. 3. Desensitization of endothelial cells to a second dose of bradykinin

Cells were loaded with fura-2 AM, placed in the spectrofluorimeter and superfused (1 ml/min) with HBSS containing 10 mM-Hepes and 0.25% BSA, pH 7.4. At 10 min, an initial dose of bradykinin (100 nM) was given under stop-flow conditions as described in Fig. 1(*a*). At 25 min, a second maximal dose of bradykinin (1 μM) was given. The dotted line represents the change in $[Ca^{2+}]_i$ from a representative of three experiments. Parallel experiments were done with cells on beads which were prelabelled with [^{3}H]arachidonic acid as described in Fig. 1(*b*). The continuous line represents arachidonic acid release data from a representative of three experiments in which an initial dose of bradykinin (100 nM) was given at 10 min, followed by a second maximal dose (1 μM) given at 25 min.

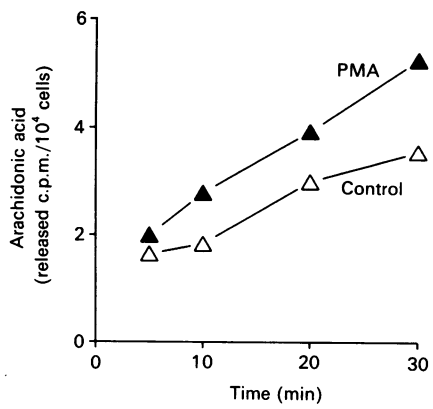

Fig. 4. Effect of PMA on arachidonic acid release from endothelial cells

Confluent monolayers of cells were prelabelled with [^{3}H]arachidonic acid as described in the Experimental section. Release of label into HBSS containing 10 mM-Hepes and 0.25% fatty-acid-free BSA, pH 7.4, was monitored over time after addition of 400 nM-PMA. Data are presented as means of duplicate samples and are representative of three similar experiments.

investigate further the dependence of arachidonic acid release on $[Ca^{2+}]_i$ in bradykinin-stimulated cells. In these experiments, cells were loaded with fura-2 ester for 1 h and were incubated with 250 μM-MAPTAM-AM for 15 min. Cells were then perfused with Ca^{2+}-free buffer containing 1 mM-EGTA. Parallel experiments were carried out with cells on beads which were prelabelled with [^{3}H]arachidonic acid and loaded with MAPTAM. As shown in Fig. 2(*a*), MAPTAM completely buffered the bradykinin-induced elevation in cytosolic Ca^{2+}. MAPTAM also prevented arachidonic acid release (Fig. 2*b*). When cells were subsequently perfused with Ca^{2+}-complete buffer, a slight increase in basal Ca^{2+} levels was observed (Fig. 2*a*), which was accompanied by a small release of labelled arachidonic acid (Fig. 2*b*). After 5 min of perfusion with Ca^{2+}-complete medium to saturate the intracellular MAPTAM with Ca^{2+}, an increase in basal $[Ca^{2+}]_i$ and a corresponding increase in arachidonic acid release were observed in response to a second dose of bradykinin. This response was somewhat attenuated, probably owing to receptor desensitization. These experiments with intracellular Ca^{2+} chelation demonstrate that a rise in $[Ca^{2+}]_i$ was required for arachidonic acid release in response to bradykinin.

A third set of experiments tested the effects of receptor desensitization on bradykinin-stimulated Ca^{2+} mobilization and arachidonic acid release. Exposure of endothelial cells to a second dose of bradykinin resulted in a comparable decrease in the Ca^{2+} response and the arachidonic acid-release response (Fig. 3). These results also demonstrate the temporal association of Ca^{2+} mobilization and arachidonic acid release.

Although the above studies strongly support Ca^{2+}-stimulated phospholipase A_2 as the mechanism for bradykinin-induced arachidonic acid release, other possibilities must be considered. Bradykinin-induced arachidonic acid release may result from protein kinase C-mediated changes in PtdIns(4,5)P_2 hydrolysis, Ca^{2+} mobilization or activation of phospholipase A_2 after diacylglycerol formation. In order to examine the role of protein kinase C, endothelial cells were treated with PMA. Endothelial cells responded to 10 min treatment with 200 nM-PMA by translocating and activating protein kinase C. Protein kinase C in the cytosol was 194 and 30 pmol of ^{32}P/min per 10^6 cells, whereas protein kinase C activity in the membrane fraction was 39 and 191 pmol of ^{32}P/min per 10^6 cells in control and PMA-treated cells respectively ($n = 2$). We also found that PMA inhibited Ca^{2+} mobilization in response to 1 μM-bradykinin. In cells pretreated for 15 min with 400 nM-PMA, peak $[Ca^{2+}]_i$ and the total Ca^{2+}

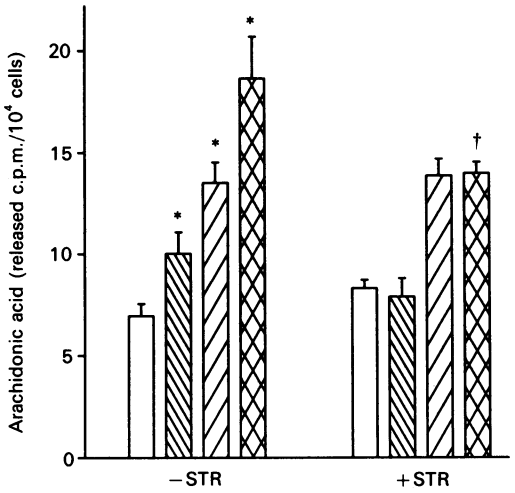

Fig. 5. Effect of PMA and bradykinin (BK) on arachidonic acid release from control or staurosporine-pretreated endothelial cells

Confluent monolayers of cells were prelabelled with [^{3}H]arachidonic acid as described in the Experimental section. After 30 min pretreatment with 0 or 50 nM-staurosporine (STR), 400 nM-PMA was added to some cultures. After an additional 30 min, cells were stimulated with BK (1 μM). Release of label was measured 5 min after addition of BK. Data are given as means ± S.E.M. ($n = 4$). Statistical significance ($P < 0.05$) is denoted by * for PMA and/or BK-treated groups versus untreated controls, and by † for staurosporine-pretreated groups versus the corresponding groups without staurosporine pretreatment. Key: □, control; ▨, PMA; ▨, BK; ▨, PMA/BK.

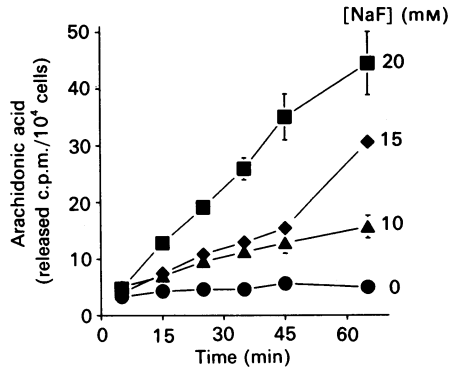

Fig. 6. Effect of different doses of NaF on arachidonic acid release from endothelial cells

Confluent monolayers of cells were prelabelled with [^{3}H]arachidonic acid as described in the Experimental section. Cells were treated with 0–20 mM-NaF plus 10 μM-$AlCl_3$, and the release of label was monitored over time. Data are given as means ± S.E.M. ($n = 3$).

transient (area under the curve) were significantly decreased by 62% and 54% respectively (results not shown; $n = 5$).

However, in endothelial cells treated with PMA alone, arachidonic acid release was stimulated (Fig. 4). Because PMA induces prostaglandin H synthase [30–32], measurement of arachidonic acid release in response to PMA was carried out in the presence of 100 μM-ibuprofen, which blocks the conversion of arachidonic acid into prostaglandins. Thus metabolites of arachidonic acid did not contribute to the label released. The effects of PMA and bradykinin on arachidonic acid release were found to be additive (Fig. 5). Pretreatment with a lower dose of PMA (25 nM for 10 min) also failed to potentiate bradykinin-

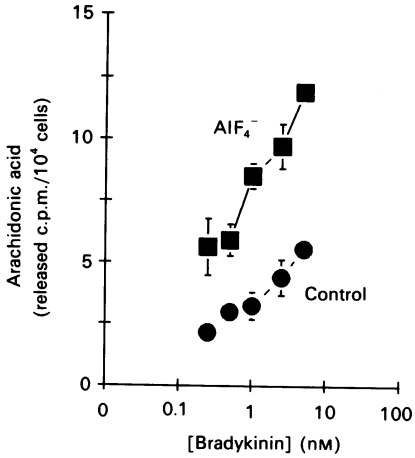

Fig. 7. Effect of AlF_4^- on bradykinin-stimulated arachidonic acid release from endothelial cells

Confluent monolayers of cells were prelabelled with [^{3}H]arachidonic acid as described in the Experimental section. After 15 min treatment with 0 or 10 mM-NaF plus 10 μM-$AlCl_3$, cells were given 2.5–50 nM-bradykinin. Release of label into medium was measured during the 5 min after addition of bradykinin. Data are given as means ± S.E.M. ($n = 4$). AlF_4^--treated groups were significantly different than controls at all bradykinin doses ($P < 0.05$). In addition, statistical analysis revealed a significant interaction between the two treatments ($P < 0.05$).

stimulated arachidonic acid release (results not shown). Furthermore, pretreatment of endothelial cells for 30 min with 50 nM-staurosporine, an inhibitor of protein kinase C, resulted in inhibition of PMA-stimulated arachidonic acid release, but not of bradykinin-induced release (Fig. 5). These experiments clearly demonstrated that protein kinase C activation can result in stimulated arachidonic acid release. However, this mechanism was not likely to account for bradykinin-induced arachidonic acid release, since the effects of PMA and bradykinin were additive and since protein kinase C inhibition had no effect on bradykinin-induced release of arachidonic acid.

We also investigated the involvement of G-proteins in bradykinin-mediated activation of phospholipase A_2. Others have demonstrated direct G-protein-mediated activation of phospholipase A_2 which is pertussis-toxin-sensitive [14–17]. Thus we used pertussis toxin as a tool to investigate the role of G-proteins in bradykinin-stimulated phospholipase A_2 activation. Endothelial cells grown in tissue-culture flasks were prelabelled with [^{3}H]arachidonic acid and incubated with 400 ng of pertussis toxin/ml for 3 h. This treatment has previously been shown by us to cause ADP-ribosylation of an approx. 42 kDa protein in endothelial-cell membranes, without altering bradykinin-stimulated Ins(1,4,5)P_3 production [5]. Pertussis-toxin treatment failed to inhibit basal or stimulated arachidonic acid release in response to 1 μM-bradykinin ($n = 4$; results not shown). Because pertussis toxin does not alter the function of all G-proteins, these negative data did not rule out a possible role of G-proteins in activating phospholipase A_2. We next examined the role of G-proteins in the bradykinin response by using AlF_4^-, a direct activator of G-proteins. Endothelial cells, which had been prelabelled with [^{3}H]arachidonic acid, were first incubated with 5–20 mM-NaF in the presence of 10 μM-$AlCl_3$. AlF_4^- stimulated significant release of arachidonic acid in a dose- and time-dependent manner (Fig. 6). NaF, but not NaCl, was also effective in stimulating arachidonic acid release, presumably owing to the presence of trace levels of Al^{3+} (results not shown). Exposures to AlF_4^- in all subsequent experiments involved 10 mM-NaF and 10 μM-$AlCl_3$.

Table 1. Effect of AlF_4^- on lysophosphatidylcholine formation

Data are presented in c.p.m., and are means ± S.E.M. ($n = 5$). Statistical significance ($P < 0.05$) is denoted by * for treated groups versus controls.

	Lysophosphatidylcholine (c.p.m.)		
Time (min)...	2.5	5.0	10.0
Control	574 ± 21	669 ± 34	681 ± 90
AlF_4^-	737 ± 46*	825 ± 18*	747 ± 67

This dose of AlF_4^- was not associated with cytotoxicity, as measured by the release of lactate dehydrogenase from cells (results not shown). In addition, treatment for 30 min with AlF_4^- enhanced prostacyclin synthesis by 2.5-fold above controls, suggesting that the released arachidonic acid was available to prostaglandin-synthetic enzymes (results not shown).

In additional experiments, endothelial cells were incubated for 15 min with AlF_4^- and challenged with 2.5–50 nM-bradykinin in the continued presence of AlF_4^- (Fig. 7). The combined treatment resulted in a greater than additive response, as indicated by the significant difference in slopes of the two curves (Fig. 7). These results suggest that G-proteins were involved in coupling the response to bradykinin or that G-protein activation modulated the response to bradykinin.

To investigate the AlF_4^--mediated response further, we also measured lysophospholipid formation. Intracellular lysophosphatidylcholine was significantly increased at 2.5 and 5 min after AlF_4^- treatment (Table 1). In contrast, formation of lysophosphatidylethanolamine and lysophosphatidylinositol was not altered by AlF_4^- treatment (results not shown). Since lysophospholipids may be formed by both phospholipase A_1 and phospholipase A_2-dependent mechanisms, we examined the release of stearic acid from prelabelled cells. Because stearic acid is normally found in the *sn*-1 position of phospholipids, enhanced release would likely reflect stimulated phospholipase A_1 activity. Results from this experiment (not shown) demonstrated only slight enhancement of stearic acid release in AlF_4^--treated cells. Hence these studies implicate phospholipase A_2 as the mechanism by which arachidonic acid release is enhanced. In addition, these studies suggest that phosphatidylcholine serves as a substrate for the phospholipase A_2-catalysed hydrolysis.

We also determined the Ca^{2+}-dependence of the AlF_4^- response by measuring arachidonic acid release under different extracellular conditions. Basal arachidonic acid release over the 25 min study period was enhanced in the absence of extracellular Ca^{2+} and Mg^{2+} by 1.9-fold, and by 2.4-fold in the presence of 1 mM-EGTA (results not shown). AlF_4^- treatment resulted in a similar degree of stimulation of arachidonic acid release (i.e. 2.0–2.1-fold above controls) in Ca^{2+}- and Mg^{2+}-complete buffer, Ca^{2+}- and Mg^{2+}- free buffer and Ca^{2+}- and Mg^{2+}-free buffer containing 1 mM-EGTA (results not shown; $n = 3$). However, the kinetics of the AlF_4^--induced response differed under these conditions, with the maximal response reached more quickly in the EGTA-containing buffer. This is in marked contrast with bradykinin-induced release, which was decreased in the absence of extracellular Ca^{2+}. Furthermore, AlF_4^--stimulated arachidonic acid release was found to be similar in Ca^{2+}-free Mg^{2+}-replete buffer and in Ca^{2+}- and Mg^{2+}-free buffer (results not shown), ruling out a possible role of Mg^{2+} in modulating this response. Finally, we examined the effects of AlF_4^- on intracellular Ca^{2+}, using fura-2-loaded cells superfused with Ca^{2+}- and Mg^{2+}-complete buffer. Although endothelial cells were responsive to 1 μM-bradykinin, treatment with AlF_4^- for 8 min failed to increase $[Ca^{2+}]_i$ (results not shown; $n = 4$).

DISCUSSION

This study demonstrates that bradykinin rapidly stimulated arachidonic acid release which was closely coupled to changes in $[Ca^{2+}]_i$ in endothelial cells. The dependence of arachidonic acid release on Ca^{2+} was demonstrated in three ways. First, we found that decreasing Ca^{2+} influx by removing Ca^{2+} from the extracellular medium resulted in decreased arachidonic acid release. Secondly, when we loaded cells with the intracellular Ca^{2+} chelator MAPTAM, the Ca^{2+} transient was buffered and arachidonic acid release was prevented. Third, when the bradykinin receptor was desensitized, we observed both a decrease in the Ca^{2+} transient and a corresponding decrease in arachidonic acid release. Taken together with previous reports by us [5] and others [6] on the production of lysophosphatides by bradykinin, these studies clearly show that changes in $[Ca^{2+}]_i$ regulate phospholipase A_2 activity. Furthermore, we found that mobilization of Ca^{2+} from intracellular stores was sufficient for phospholipase A_2 activation, although it did not support a full response. This is in contrast with Brooks *et al.* [33], who reported that phospholipase A_2 activation was completely dependent on the influx of extracellular Ca^{2+} in rat glioma cells. It should be noted that measurements of arachidonic acid release in this latter study were conducted under static-flow conditions.

Although Ca^{2+} is clearly involved in bradykinin-stimulated arachidonic acid release, other mediators produced in bradykinin-stimulated cells might modulate this response. For example, diacylglycerol formed in bradykinin-stimulated cells may lead to protein kinase C activation. However, our results do not support a role for protein kinase C in bradykinin-stimulated arachidonic acid release for two reasons. First, protein kinase C inhibition was ineffective in preventing arachidonic acid release in bradykinin-stimulated endothelial cells. Secondly, pretreatment with protein kinase C activator PMA failed to potentiate bradykinin-stimulated arachidonic acid release. However, we found that PMA inhibited bradykinin-stimulated Ca^{2+} mobilization and that it stimulated arachidonic acid release on its own. Phorbol esters have similar effects on Ca^{2+} mobilization in human umbilical-vein endothelium [13,34] and on arachidonic acid release in MDCK cells [35,36]. We conclude that, although phospholipase A_2 activation may occur secondarily to protein kinase C activation, protein kinase C was not involved in bradykinin-induced arachidonic acid release. Hence our results do not support the proposal that protein kinase C plays a role in Ca^{2+}-dependent agonist activation of phospholipase A_2 [12,35]. Similar findings have been reported for ATP-induced prostacyclin production [13].

Direct coupling of agonist receptors to phospholipase A_2 via G proteins has been reported in noradrenaline-stimulated thyroid cells [14], light-activated rod outer segments [15,16] and epidermal-growth-factor-stimulated renal tubule cells [17]. We have demonstrated phospholipase A_2 activation in pig aortic endothelial cells, using the G-protein activator AlF_4^-. In these studies, we found significant stimulation of arachidonic acid release and increased production of its vasodilatory metabolite, prostacyclin. Interestingly, two recent reports describe AlF_4^--stimulated vasodilation in coronary-artery rings [37,38]. We also found that AlF_4^- treatment increased the formation of lysophosphatidylcholine, implicating phosphatidylcholine-specific phospholipase A_2 as the mechanism by which arachidonic acid is released. Importantly, we demonstrated that AlF_4^--stimulated arachidonic acid release did not require the presence of extra-

cellular Ca^{2+} or the mobilization of intracellular Ca^{2+}, in distinct contrast with the bradykinin response, which we showed to be highly dependent on $[Ca^{2+}]_i$. This Ca^{2+}-independent AlF_4^--stimulated phospholipase A_2 represents a novel pathway for arachidonic acid release.

We also found that AlF_4^- enhanced bradykinin-stimulated arachidonic acid release. This finding suggests either that G-proteins are directly involved in coupling the bradykinin response or that G-protein activation modulates the bradykinin response. G-protein-mediated coupling of the bradykinin receptor to phospholipase A_2 is unlikely, given the inability of bradykinin to stimulate arachidonic acid release under conditions where no mobilization of intracellular Ca^{2+} or influx of extracellular Ca^{2+} occurred. Alternatively, G-proteins may directly couple the bradykinin receptor to phospholipase C. However, our studies do not provide evidence for this, since AlF_4^- failed to mobilize $[Ca^{2+}]_i$. These results do not rule out G-protein-mediated coupling of the bradykinin receptor to phospholipase C, since AlF_4^- preferentially activates G_i, the pertussis-toxin-sensitive G-protein, in intact cell preparations [39], and other G-proteins may be involved in the response. In fact, phospholipase C activation is mediated by pertussis-toxin-insensitive G proteins in ATP- and thrombin-stimulated endothelial cells [40–44]. Similarly, pertussis toxin failed to block bradykinin-stimulated arachidonic acid release in the present study and Ins(1,4,5)P_3 formation in a previous study [5]. Hence, the effects of AlF_4^- were probably due to modulation of bradykinin-stimulated arachidonic acid release. This may have resulted from an enhanced sensitivity of phospholipase A_2 to Ca^{2+}, since G-protein activation lowers the Ca^{2+} requirement of agonist-stimulated phospholipase A_2 [45].

To our knowledge, this is the first report of Ca^{2+}-dependent and Ca^{2+}-independent pathways of phospholipase A_2 activation in intact cells. These findings are significant in that they point to multiple pathways of phospholipase A_2 activation which may operate in parallel in the pig aortic endothelial cell. Whether these pathways involve the same phospholipase A_2, which is regulated differentially by Ca^{2+}, protein kinase C and G-proteins, or separate enzyme proteins with their own regulatory mechanisms is yet to be determined.

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