Ca²⁺-independent arachidonic acid release by vascular endothelium requires protein synthesis *de novo*

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We investigated the mechanism by which the G-protein activators aluminium fluoride and vanadate stimulate arachidonic acid release in pig aortic endothelial cells. Our previous study demonstrated a novel Ca²⁺-independent pathway of phospholipase A₂ (PLA₂) activation stimulated by aluminium fluoride in this model. In the present study, we found that sodium metavanadate stimulated a rapid concentration-dependent release of [³H]-arachidonic acid from prelabelled cells. A more than 3-fold enhancement of arachidonic acid release was achieved in cells treated with 1 mM vanadate for 20 min. Synthesis of prostaglandin products was similarly enhanced. The release of arachidonic acid was not dependent on the presence of extracellular Ca²⁺, but did require protein synthesis *de novo*. Both cycloheximide and actinomycin D completely blocked aluminium fluoride- and vanadate-stimulated arachidonic acid release.

Because fluoride and vanadate are known protein tyrosine phosphatase inhibitors, it is possible that PLA₂ activation occurred secondarily to changes in protein tyrosine phosphorylation. Both aluminium fluoride and vanadate stimulated the rapid phosphorylation of 58, 93 and 120 kDa tyrosine-containing protein substrates. However, in contrast with arachidonic acid release, this response was found to be sensitive to the presence of extracellular Ca²⁺ and insensitive to blockers of protein synthesis *de novo*. Furthermore, H₂O₂ treatment resulted in rapid tyrosine phosphorylation of the same substrates without a concomitant increase in arachidonic acid release. These results suggest that the effects of aluminium fluoride and vanadate on PLA₂ are not due to changes in protein tyrosine phosphorylation, but do require rapid protein synthesis *de novo*.

INTRODUCTION

Phospholipases A₂ (PLA₂) are a class of enzymes which catalyse the hydrolysis of arachidonic acid and other unsaturated fatty acids from the *sn*-2 position of phospholipids [1–3]. In vascular endothelium and other cell types, the availability of unesterified arachidonic acid is the rate-limiting step in the formation of eicosanoids [1–3]. Prostacyclin and prostaglandin E₂ are the major eicosanoids produced by vascular endothelial cells, and they play a key role in modulating vasomotor tone, platelet function and inflammation [4–7]. Despite numerous studies, the regulation of PLA₂ in endothelial and other cells is incompletely understood. Three PLA₂ isoforms have been identified in mammalian cells: Type I and Type II 14 kDa secretory enzymes, and an 85 kDa enzyme [2,3]. All three exhibit sensitivity to Ca²⁺ [2,3,8–10]. Millimolar concentrations of Ca²⁺ are required for catalysis of the 14 kDa enzymes [2,3,8], whereas micromolar concentrations are required for translocation of the 85 kDa enzyme from cytosol to membrane [9,10]. Differences in specificity for unsaturated fatty acids present in the *sn*-2 position have been observed for the three isoforms, with only the 85 kDa enzyme showing specificity for arachidonate-containing phospholipids [10,11]. Differences in distribution of the three isoforms have also been observed. The Type I 14 kDa enzyme is mainly found in the pancreas and in pancreatic secretions [12], whereas the Type II 14 kDa enzyme is found intracellularly in several cell types, including platelets [13], leucocytes [14], astrocytes [15], chondrocytes [16] and hepatoma cells [17] and extracellularly in inflammatory exudates [14], synovial fluid [18] and serum [14]. The 85 kDa enzyme is found intracellularly in monocyte/macrophage cell lines [9,10], platelets [19] and neutrophils [19], and in several tissues including brain, lung and liver

[19]. However, little is known about the relative distributions of the various isoforms and their relative contributions to arachidonic acid release and eicosanoid generation in most cell types. Recently the presence of both Type II 14 kDa and the 85 kDa enzymes was demonstrated in human umbilical vein endothelium [20]. Both types of enzymes were activated by Ca²⁺-mobilizing agonists and participated in eicosanoid generation [20].

In addition to Ca²⁺-dependent processes, other cell signalling pathways play a role in modulating PLA₂ activity in mammalian cells. Regulation by protein kinase C and other kinases [21–24], G-proteins [25–28] and transcriptional events [15–17,29,30] occurs in several systems. In addition, Ca²⁺-independent PLA₂ activities are found in myocardial [31] and lung [32] cytosol and in lysates of pancreatic β cells [33]. We have recently reported a Ca²⁺-independent pathway of PLA₂ activation in pig aortic endothelium stimulated with the G-protein activator aluminium fluoride [34]. These results are novel in that they demonstrate Ca²⁺-independent PLA₂ activation in an intact cell type. Our preliminary data also demonstrate stimulated arachidonic acid release in response to vanadate, another G-protein activator [35]. Although G-proteins may be involved in these responses by directly activating PLA₂, other possibilities exist. For example, G-protein-mediated PLA₂ activation requires protein synthesis *de novo* in some cases [29,30]. Furthermore, aluminium fluoride and vanadate are known to inhibit protein tyrosine phosphatases [36]. Although there is no direct evidence of PLA₂ regulation by protein tyrosine phosphorylation, PLA₂ activity is stimulated by growth factors coupled to receptor tyrosine kinases in several cell types [37,38]. For these reasons, the goal of the present study was to investigate the mechanism by which aluminium fluoride and vanadate stimulate arachidonic acid release in pig aortic endo-

Abbreviations used: PLA₂, phospholipase A₂; DMEM, Dulbecco's modified Eagle medium; HHBSS, Hanks' balanced salt solution containing 10 mM Hepes, pH 7.4.

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thelium. Specifically, we examined the role of protein synthesis *de novo* and protein tyrosine phosphorylation in mediating this response. Our results demonstrate that Ca^{2+} -independent PLA_2 activation in our model involves protein synthesis *de novo*, but not protein tyrosine phosphorylation.

EXPERIMENTAL

Materials

[^3H]Arachidonic acid was from New England Nuclear (Wilmington, DE, U.S.A.) and affinity-purified [^{125}I]-Protein A was from Amersham. Cell culture reagents were from GIBCO (Grand Island, NY, U.S.A.). Tissue-culture plasticware was from NUNC (Scientific Technologies, Raleigh, NC, U.S.A.). Reagents used in determination of prostaglandin production and in protein analysis by the bicinchoninic acid method were obtained from Pierce (Rockford, IL, U.S.A.). Affinity-purified polyclonal antisera to phosphotyrosine were kindly provided by Dr. Dierdre Luttrell of Glaxo (Research Triangle Park, NC, U.S.A.). Buffers for PAGE and Western protein analysis and Kodak X-Omat AR autoradiography film were obtained from Eastman Kodak (Rochester, NY, U.S.A.). Biotinylated molecular-mass markers were obtained from Bio-Rad Laboratories (Hercules, CA, U.S.A.). All other reagents were from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell culture

Endothelial cells were isolated and cultured by established methods as previously described [34]. Briefly, pig aortas were enzymically digested with collagenase, and isolates were plated on tissue-culture plasticware (NUNC). Cells were incubated at 37 °C in an atmosphere of 10% CO_2 in air in Dulbecco's Modified Eagle Medium (DMEM) containing 10% (v/v) fetal-bovine serum and antibiotic/antimycotic (100 units/ml penicillin G sodium, 100 $\mu\text{g}/\text{ml}$ streptomycin sulphate and 250 ng/ml amphotericin B). 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 [34]. Primary cells and cells in passages 1–2 were used in these studies.

Arachidonic acid release and prostaglandin production

To measure arachidonic acid release, confluent monolayers of endothelial cells were labelled by overnight incubation with 0.4 $\mu\text{Ci}/\text{ml}$ [^3H]arachidonic acid in DMEM containing 0.5–1% bovine serum and antibiotic/antimycotic. Unincorporated label was removed by washing with $4 \times 5 \text{ ml}$ of Hanks' balanced salt solution containing 10 mM Hepes, pH 7.4 (HHBSS). Cells in T-25 flasks (approx. 0.8 mg of protein per flask) were stimulated with various agents at 37 °C for the indicated times in 5 ml of HHBSS containing 0.25% fatty-acid-free BSA. The release of labelled fatty acid was measured by liquid-scintillation spectroscopy. Prostaglandin products, prostacyclin and prostaglandin E_2 , 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 [39]. The major prostaglandin product, prostacyclin, was measured as its stable breakdown product 6-oxo-prostaglandin $\text{F}_{1\alpha}$.

Immunoblot procedures for protein tyrosine phosphorylation

Cells were incubated overnight in DMEM containing 0.5–1% fetal-bovine serum and antibiotic/antimycotic, to decrease

serum-stimulated protein tyrosine phosphorylation. Cells in T-25 flasks were washed free of culture medium by using HHBSS and stimulated with various agents at 37 °C for the indicated times in HHBSS. After removal of the buffer, cells were lysed with 0.5 ml of boiling 63 mM Tris, pH 6.8, containing 10% (v/v) glycerol and 2% (w/v) SDS. After scraping of the monolayer, lysates were sonicated and a portion was removed for protein analysis by the bicinchoninic acid method. After addition of 2-mercaptoethanol and Bromophenol Blue to final concentrations of 5% (v/v) and 0.002% (w/v) respectively, the lysates were boiled for 5 min at 100 °C and frozen at -20°C until analysis. Proteins were separated by SDS/PAGE on 8%-acrylamide minigels in 0.025 M Tris buffer, pH 8.3, containing 0.192 M glycine and 0.1% SDS and 15 μg of protein per lane. Proteins were then stained with Coomassie Blue or transferred to nitrocellulose in the presence of 0.025 M Tris buffer, pH 8.3, containing 0.192 M glycine, 0.075% (w/v) SDS, 0.5 mM sodium orthovanadate and 20% (v/v) methanol. Blots were blocked overnight in 100 mM Tris, pH 7.2, containing 150 mM NaCl, 0.05% Nonidet P-40, 0.05% Tween 20, 4% BSA and 0.01% NaN_3 . After rinsing with the same buffer in the absence of NaN_3 and BSA, blots were incubated with 1 $\mu\text{g}/\text{ml}$ affinity-purified polyclonal rabbit antisera to phosphotyrosine in blocking buffer. After rinsing, blots were incubated for 90 min in 0.5 $\mu\text{Ci}/\text{ml}$ affinity-purified [^{125}I]-Protein A. After rinsing, blots were dried in room air and subjected to autoradiography using Kodak X-OMAT AR film. Blocking control experiments involved the prior incubation of undiluted antibody with 80 mM phosphotyrosine for 1 h in blocking buffer, followed by dilution of antibody to its working concentration and incubation with the blot in the presence of 5 mM phosphotyrosine for 3 h. Estimation of molecular masses of phosphotyrosine-containing proteins was based on prestained and biotinylated molecular-mass markers transferred to nitrocellulose.

Cytotoxicity

Cytotoxicity was determined by quantifying the amount of lactate dehydrogenase released by cells during incubation in HHBSS in the presence and absence of sodium metavanadate. The amount of lactate dehydrogenase released was compared with the amount contained in cell lysates prepared by solubilizing and sonication in HHBSS containing 0.05% Triton X-100. Lactate dehydrogenase was determined by spectrophotometric assay [40].

RESULTS

Our previous study in pig aortic endothelial cells demonstrated Ca^{2+} -dependent PLA_2 activation in response to the Ca^{2+} -mobilizing agonist bradykinin and Ca^{2+} -independent PLA_2 activation in response to the G-protein activator aluminium fluoride [34]. In a preliminary study, we found that another G-protein activator, sodium metavanadate, stimulated the release of arachidonic acid in this same model [35]. In order to examine the time- and concentration-dependence of this response, we prelabelled endothelial cells with [^3H]arachidonic acid and exposed them to 0.05–1 mM sodium metavanadate for 20 min. Stimulated arachidonic acid release was observed as early as 2.5 min after treatment with 0.1–1 mM vanadate (Figure 1). Treatment with 1 mM vanadate for 20 min resulted in a more than 3-fold stimulation of arachidonic acid release. These doses of vanadate were not associated with cytotoxicity as measured by the release of lactate dehydrogenase from cells (results not shown). In addition, treatment with 1 mM vanadate for 30 min enhanced prostacyclin

Figure 1 Effects of sodium metavanadate on arachidonic acid release from endothelial cells

Confluent monolayers of cells were prelabelled with [³H]arachidonic acid as described in the Experimental section. Cells were treated with 0–1 mM sodium metavanadate (Van) in the presence of HHBSS and 0.25% fatty-acid-free BSA. The release of label was monitored over time. Data are given as the averages of samples in duplicate.

Figure 2 Effects of extracellular Ca²⁺ on vanadate-stimulated arachidonic acid release from endothelial cells

Confluent monolayers of cells were prelabelled with [³H]arachidonic acid as described in the Experimental section. After rinsing three times with Ca²⁺-free HHBSS containing 1 mM EGTA (○, ●) or Ca²⁺-free HHBSS (□, ■), cells were treated with 0 (Con) or 1 mM sodium metavanadate (Van) in the appropriate buffer containing 0.25% fatty-acid-free BSA. The release of label was monitored over time. Data are given as means ± S.E.M. (*n* = 3).

synthesis from 16.3 ± 3.5 ng in controls to 70.3 ± 3.9 ng (*n* = 3) and enhanced prostaglandin E₂ synthesis from 0.5 ± 0.2 ng in controls to 2.9 ± 0.3 ng (*n* = 3). These results indicate that the arachidonic acid which was released was available for prostaglandin synthesis. We have reported similar responses to aluminium fluoride in this model [34].

We next determined the Ca²⁺-dependence of the vanadate response by measuring arachidonic acid release under conditions of low extracellular Ca²⁺. Our previous study demonstrated that depletion of extracellular Ca²⁺ inhibited arachidonic acid release in endothelial cells exposed to Ca²⁺-mobilizing agonists such as bradykinin [34]. Figure 2 demonstrates the stimulation of arachidonic acid release by vanadate from prelabelled cells which were incubated in nominally Ca²⁺-free buffer and EGTA-con-

Figure 3 Effect of cycloheximide on aluminium fluoride- and vanadate-stimulated arachidonic acid release

Confluent monolayers of cells were prelabelled with [³H]arachidonic acid as described in the Experimental section. Cells were treated for 10 min with HHBSS (Con) or HHBSS containing 5 μg/ml cycloheximide (Cyc) before and during incubation with (a) aluminium fluoride (AlF₄⁻; 10 mM NaF plus 10 μM AlCl₃) or (b) 1 mM sodium metavanadate (Van) in the presence of 0.25% fatty-acid-free BSA. The release of label was monitored over time. Data are given as means ± S.E.M. (*n* = 3).

taining buffer. Incubation for 20 min with 1 mM sodium metavanadate in the absence of extracellular Ca²⁺ resulted in a 5–6-fold stimulation of arachidonic acid release. A similar incubation with vanadate in the presence of extracellular Ca²⁺ resulted in a 2-fold stimulation of arachidonic acid release in this study (results not shown). Hence, vanadate-stimulated arachidonic acid release was not inhibited by the removal of extracellular Ca²⁺. Aluminium fluoride-stimulated arachidonic acid release also occurred in the absence of extracellular Ca²⁺ and did not require the mobilization of intracellular Ca²⁺ [34].

These results suggest a Ca²⁺-independent mechanism by which G-protein activators modulate PLA₂ activity in pig aortic endothelium. Others have demonstrated a G-protein-coupled pathway of PLA₂ activation which involves the synthesis of an activating protein [29,30]. For this reason, we wished to determine whether the responses to aluminium fluoride and vanadate were under transcriptional or translational control. Figure 3 demonstrates the release of arachidonic acid in cells treated with 5 μg/ml of the protein-synthesis inhibitor cycloheximide for 10 min before exposure to aluminium fluoride or vanadate. Aluminium fluoride- and vanadate-stimulated arachidonic acid release was effectively blocked by cycloheximide. Similarly, 30 min preincubation with 5 μg/ml of the RNA-synthesis inhibitor actinomycin D resulted in inhibition of aluminium

Figure 4 Effect of actinomycin D on aluminium fluoride- and vanadate-stimulated arachidonic acid release

Confluent monolayers of cells were prelabelled with [³H]arachidonic acid as described in the Experimental section. Cells were treated for 30 min with HHBSS (Con) or with HHBSS containing 5 µg/ml actinomycin D (Act) before and during incubation with aluminium fluoride (AlF₄⁻; 10 mM NaF plus 10 µM AlCl₃) or 1 mM sodium metavanadate (Van) in the presence of 0.25% fatty-acid-free BSA. The release of label was monitored over time. Data are given as means ± S.E.M. (n = 3).

Figure 5 Effect of vanadate on protein tyrosine phosphorylation in endothelial cells

Confluent monolayers of endothelial cells were incubated with 1 mM sodium metavanadate for the indicated times (in min) or buffer alone for 16 min (CON), and the reaction was quickly stopped as described in the Experimental section. Samples were subjected to SDS/PAGE on 8% acrylamide gels and transferred to nitrocellulose. Immunoblotting procedures were carried out with affinity-purified polyclonal rabbit antisera to phosphotyrosine and affinity-purified ¹²⁵I-Protein A, as described in the text. The approximate molecular masses (kDa) of tyrosine-containing substrates are presented on the left, and are based on comparisons with molecular-mass markers run in parallel.

fluoride- and vanadate-stimulated arachidonic acid release (Figure 4). These findings indicate that the mechanism by which aluminium fluoride and vanadate stimulate Ca²⁺-independent PLA₂ activity in pig aortic endothelium requires protein synthesis *de novo*.

Besides activating G-proteins, fluoride and vanadate are known to inhibit protein tyrosine phosphatases [36]. These effects result from the ability of these compounds to mimic the γ-phosphate groups of GTP and ATP, thus blocking phosphate-transfer reactions [41]. It is not known whether protein tyrosine phosphorylation plays a role in regulating PLA₂. However, protein tyrosine phosphorylation has been linked to protein synthesis *de novo* [42,43] and PLA₂ activity is stimulated by growth factors coupled to receptor tyrosine kinases in several cell types [37,38]. For these reasons, we investigated the role of

Figure 6 Effect of extracellular Ca²⁺ on fluoride- and vanadate-stimulated protein tyrosine phosphorylation

Confluent monolayers of endothelial cells were rinsed three times with Ca²⁺-free HHBSS containing 1 mM EGTA (EGTA), Ca²⁺-free HHBSS (−Ca²⁺) or HHBSS (+Ca²⁺) and incubated for 5 min in the appropriate buffer alone (Con) or buffer containing (a) 1 mM sodium metavanadate (Van) or (b) aluminium fluoride (AlF₄⁻). The reaction was quickly terminated, and samples were processed for SDS/PAGE and immunoblotting as described in the text.

protein tyrosine phosphorylation in stimulating arachidonic acid release in aluminium fluoride- and vanadate-stimulated cells. Figure 5 demonstrates protein tyrosine phosphorylation in endothelial cells exposed to vanadate for up to 16 min. The major substrates, 58, 93 and 120 kDa proteins, were phosphorylated in a time-dependent fashion, with an increase in the degree of phosphorylation observed as early as 0.5 min, reaching a peak by 4 min and remaining above basal levels for as long as 16 min. Similar findings were obtained in aluminium fluoride-treated cells (results not shown), although the magnitude of the response was not as great (Figures 6 and 7). No protein tyrosine phosphorylation was observed in blocking control experiments using phosphotyrosine (results not shown). Vanadate- and aluminium fluoride-dependent phosphorylation of serine- and threonine-containing proteins was not tested, since inhibitors of serine and threonine specific phosphatases, okadaic acid and calyculin A, did not stimulate arachidonic acid release in prelabelled cells (results not shown).

In order to probe the relationship between protein tyrosine phosphorylation and arachidonic acid release, we used different levels of extracellular Ca²⁺ and blockers of protein synthesis

Figure 7 Effect of cycloheximide and actinomycin D on fluoride- and vanadate-stimulated protein tyrosine phosphorylation

Confluent monolayers of endothelial cells were preincubated (a) for 10 min with 5 μ g/ml cycloheximide (Cyc) in HHBSS or (b) for 30 min with 5 μ g/ml actinomycin D (Act) in HHBSS and treated with buffer alone (Con) or with 1 mM sodium metavanadate (Van) or aluminium fluoride (AlF_4^-) for 5 min in HHBSS. The reaction was quickly terminated, and samples were processed for SDS/PAGE and immunoblotting as described in the text.

and RNA synthesis to see if alterations in protein tyrosine phosphorylation and arachidonic acid release occurred in parallel. In one study, we determined protein tyrosine phosphorylation in endothelial cells incubated with or without extracellular Ca^{2+} . Basal phosphorylation was decreased considerably under nominally Ca^{2+} -free conditions and in the presence of 1 mM EGTA, compared with controls (Figure 6). Protein tyrosine phosphorylation was stimulated by 5 min incubation with vanadate (Figure 6a), and to a lesser extent by 5 min incubation with aluminium fluoride (Figure 6b) under all conditions of extracellular Ca^{2+} . However, the responses stimulated by aluminium fluoride and vanadate were diminished in the absence of extracellular Ca^{2+} , both in nominally Ca^{2+} -free buffer and in buffer containing the Ca^{2+} chelator EGTA. These conditions of decreased extracellular Ca^{2+} failed to decrease basal or stimulated arachidonic acid release (Figure 2). Hence, aluminium fluoride- and vanadate-stimulated protein tyrosine phosphorylation and arachidonic acid release differ in their dependence on extracellular Ca^{2+} .

In a second set of studies, we determined protein tyrosine phosphorylation in aluminium fluoride- and vanadate-treated

Figure 8 Effect of H_2O_2 on protein tyrosine phosphorylation in endothelial cells

Confluent monolayers of cells were incubated with buffer alone (Con) or with 100 μ M H_2O_2 in HHBSS for the indicated times. The reaction was quickly terminated, and samples were processed for SDS/PAGE and immunoblotting as described in the text. The 'kDa' values are as for Figure 5.

Figure 9 Effect of H_2O_2 on arachidonic acid release from endothelial cells

Confluent monolayers of cells were prelabelled with [^3H]arachidonic acid as described in the Experimental section. Cells were treated with buffer alone (Con) or with 100 μ M H_2O_2 , aluminium fluoride (AlF_4^-) or 1 mM sodium metavanadate (Van) in HHBSS containing 0.25% fatty-acid-free BSA. The release of label was monitored over time. Data are given as means $\pm$ S.E.M. ($n = 3$).

cells which were preincubated with inhibitors of protein synthesis and RNA synthesis (Figure 7). Preincubation for 10 min with 5 μ g/ml cycloheximide resulted in a slight inhibition of protein tyrosine phosphorylation in response to aluminium fluoride and vanadate (Figure 7a). Preincubation for 30 min with 5 μ g/ml actinomycin D resulted in partial inhibition of basal and stimulated protein tyrosine phosphorylation (Figure 7b). In contrast, inhibition of protein and RNA synthesis effectively blocked arachidonic acid release stimulated by aluminium fluoride and vanadate (Figure 4). This experiment also suggests that protein tyrosine phosphorylation and arachidonic acid release occur by separate mechanisms.

To test further the dependence of arachidonic acid release on protein tyrosine phosphorylation, we incubated cells with another agent reported to inhibit protein tyrosine phosphatases [44]. Exposure of endothelial cells to 100 μ M H_2O_2 for 60 min resulted in the time-dependent phosphorylation of 58, 93 and 120 kDa tyrosine-containing proteins (Figure 8). We have previously shown this exposure to be sub-lethal in this model [34]. However,

incubation with H_2O_2 for 60 min had only a small effect on arachidonic acid release (Figure 9). Thus it appears that phosphorylation of the 58, 93 and 120 kDa protein substrates is not sufficient for PLA_2 activation.

DISCUSSION

The focus of this investigation was on a novel Ca^{2+} -independent pathway of PLA_2 activation observed in intact pig aortic endothelial cells. Our previous study demonstrated Ca^{2+} -independent arachidonic acid release in cells treated with the G-protein activator aluminium fluoride [34]. This response was accompanied by increased formation of lysophosphatidylcholine, thus providing evidence for PLA_2 activation by aluminium fluoride. In the present study we found that this pathway is stimulated by vanadate and that it requires protein synthesis *de novo*. These findings have several important implications.

First, since both aluminium fluoride and vanadate are G-protein activators [45], our results suggest a role for G-proteins in coupling the response. Direct coupling of PLA_2 to G-protein-linked receptors has been reported in epidermal-growth-factor-stimulated renal tubule cells [28] and noradrenaline-stimulated thyroid cells [25]. Furthermore, direct activation of PLA_2 by the $\beta\gamma$ subunits of G-proteins has been demonstrated in the rod outer segment [26,27]. Indirect coupling of G-protein-linked receptors to PLA_2 activation via phospholipase C and the subsequent generation of intracellular messengers such as Ca^{2+} has been demonstrated in many systems. However, this indirect mechanism is not likely to be involved in our model, since aluminium fluoride treatment does not result in increased intracellular Ca^{2+} or inositol trisphosphate formation, and since extracellular Ca^{2+} is not required for the response to aluminium fluoride [34] or vanadate.

Secondly, our results suggest that PLA_2 is regulated by transcriptional and translational events in our model. The ability of cycloheximide and actinomycin D to block completely aluminium fluoride- and vanadate-stimulated arachidonic acid release suggests that either the enzyme itself or an activating factor is quickly up-regulated in response to these agents. Given the rapidity of the arachidonic acid release response, which is increased within 2.5 min after vanadate addition, such a regulatory process would have to occur in the time frame of seconds to minutes. Although cytokine treatment results in the increased synthesis of the Type II 14 kDa PLA_2 isoform, this response occurs slowly, requiring a minimum of several hours before significant changes are observed [15–17]. A more likely candidate for upregulation is PLA_2 -activating protein (PLAP), which has been demonstrated in several cell types [29,30]. Increased PLAP mRNA and immunoreactive protein occurred minutes after the addition of leukotriene D_4 or tumour necrosis factor to bovine pulmonary-artery endothelial cells [29,30]. This response was coupled through G-proteins, since pertussis-toxin pretreatment blocked the response to tumour necrosis factor [29]. Results of our present study are consistent with the hypothesis that synthesis *de novo* of PLAP mediates G-protein-coupled PLA_2 activation and that this occurs without a requirement for Ca^{2+} as a second messenger. This possibility has not yet been tested.

Furthermore, our results are of interest because they indicate that protein tyrosine phosphorylation is not responsible for the stimulated arachidonic acid release observed in our model. Since aluminium fluoride and vanadate are effective inhibitors of protein tyrosine phosphatases, treatment with these agents would be expected to result in increased phosphorylation of tyrosine-containing proteins. We observed a rapid time-dependent increase in protein tyrosine phosphorylation of several substrates,

most notably 58, 93 and 120 kDa proteins, in aluminium fluoride- and vanadate-treated cells. Although the identities of these protein substrates are not known, their phosphorylation probably results from basal activity of endogenous protein tyrosine kinases in the pig aortic endothelial cell. In fact, treatment with the protein tyrosine kinase inhibitor genistein resulted in decreased basal and vanadate-stimulated protein tyrosine phosphorylation (results not shown). Our conclusion that protein tyrosine phosphorylation does not play a role in vanadate- and aluminium fluoride-stimulated arachidonic acid release is supported by three lines of evidence. First of all, basal and stimulated protein tyrosine phosphorylation was dependent on the presence of extracellular Ca^{2+} , whereas basal and stimulated arachidonic acid release did not require the presence of extracellular Ca^{2+} . Secondly, cycloheximide and actinomycin D had small inhibitory effects on basal and stimulated protein tyrosine phosphorylation, whereas treatment with these agents completely blocked aluminium fluoride- and vanadate-stimulated release. Thirdly, treatment with H_2O_2 resulted in a time-dependent increase in protein tyrosine phosphorylation of the 58, 93 and 120 kDa proteins, without a concomitant stimulation of arachidonic acid release. Hence protein tyrosine phosphorylation of these protein substrates is not a sufficient signal for the arachidonic acid release response.

In summary, our results suggest the presence of a G-protein-linked Ca^{2+} -independent pathway of PLA_2 activation in pig aortic endothelial cells which requires protein synthesis *de novo*. These findings are significant, because they point to a mechanism by which G-protein activation leads to protein synthesis *de novo* and thus to PLA_2 activation. The cell signalling pathways involved in coupling G-proteins and transcriptional/translational events in this model are not known. Protein kinase activation may be involved, although a role for protein tyrosine phosphorylation is not likely. Furthermore, it is not known which PLA_2 isoforms are activated by this Ca^{2+} -independent pathway. Future studies will be required to investigate these important issues.

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