Biochem. J. (1989) 264, 703–711 (Printed in Great Britain)

Endothelial inositol phosphate generation and prostacyclin production in response to G-protein activation by AlF_4^-

Magnús K. MAGNÚSSON,*§ Haraldur HALLDÓRSSON,*† Matthías KJELD‡ and Gudmundur THORGEIRSSON*†

*Department of Pharmacology, University of Iceland, Reykjavík, and Departments of †Medicine and ‡Clinical Chemistry, Landspítalinn, University Hospital, Reykjavík, Iceland

In order to elucidate the role of guanine-nucleotide-binding proteins (G-proteins) in endothelial prostacyclin (PGI_2) production, human umbilical vein endothelial cells, prelabelled with either [^{3}H]inositol or [^{3}H]arachidonic acid, were stimulated with the non-specific G-protein activator aluminium fluoride (AlF_4^-). AlF_4^- caused a dose- and time-dependent generation of inositol phosphates, release of arachidonic acid and production of PGI_2. The curves for the three events were similar. When the cells were stimulated in low extracellular calcium (60 nM), they released [^{3}H]arachidonic acid and produced PGI_2, but depleting the intracellular Ca^{2+} stores by pretreatment with the Ca^{2+} ionophore A23187 totally inhibited both events, although the cells still responded when extracellular Ca^{2+} was added. The Ca^{2+} ionophore did not inhibit the generation of inositol phosphates in cells maintained at low extracellular Ca^{2+}. Pertussis toxin pretreatment (14 h) altered neither inositol phosphate nor PGI_2 production in response to AlF_4^-. To investigate the functional role of the diacylglycerol/protein kinase C arm of the phosphoinositide system, the cells were pretreated with the protein kinase C activator 12-*O*-tetradecanoylphorbol 13-acetate (TPA) or the protein kinase C inhibitor 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H7). TPA inhibited the AlF_4^--induced inositol phosphate generation but stimulated both the release of arachidonic acid and the production of PGI_2. H7 had opposite effects both on inositol phosphate generation and on PGI_2 production. These results suggest that AlF_4^--induced PGI_2 production is mediated by a pertussis-toxin-insensitive G-protein which activates the phosphoinositide second messenger system. This production of PGI_2 can be modulated by protein kinase C activation, both at the level of inositol phosphate generation and at the level of arachidonic acid release.

INTRODUCTION

The phosphoinositide second messenger system has been shown to be a regulator of a variety of cell functions, including endothelial prostacyclin (prostaglandin I_2; PGI_2) production [1–3]. The binding of an agonist to a receptor on the cell surface activates phospholipase C which then splits phosphatidylinositol bisphosphate into the two second messengers, inositol trisphosphate and diacylglycerol. Inositol trisphophate releases Ca^{2+} from intracellular stores, while diacylglycerol activates protein kinase C which phosphorylates several cell proteins [4,5].

In endothelial cells, agonist-stimulated PGI_2 production has been shown to closely parallel inositol phosphate generation [1,2]. PGI_2 production has also been shown to be dependent on intracellular Ca^{2+} stores [6] suggesting an important regulatory role for the inositol trisphosphate arm of the phosphoinositide system. Diacylglycerol is also formed after receptor activation in these cells [7], but less is known about the role of this system in the production of PGI_2. Pretreatment with phorbol ester, a protein kinase C activator, inhibits receptor-activated inositol phosphate generation and Ca^{2+} release in endothelial cells [1,8], but the effect on the PGI_2 production is more complex and depends both on the concentration of the agonist and on the duration of phorbol ester pretreatment [1].

The existence of guanine-nucleotide-binding proteins (G-proteins), which couple receptors to their effector enzymes, is well established in the cyclic AMP system and in the activation by light of the retinal cyclic GMP phosphodiesterase. The G-proteins of those systems have been isolated and cloned [9]. Receptor coupling to phospholipase C has also been suggested to be a G-protein-mediated process [10]. In several cell types, known G-protein activators have been shown to induce inositol phosphate generation [11–13]. Judging from the sensitivity to pertussis toxin, an inhibitor of G_i, the G-proteins involved in this activation seem to vary between different cell types [14,15]. In endothelial cells pertussis toxin has been shown to inhibit ATP-induced inositol phosphate generation [16], while having no effect on such generation in response to bradykinin [17], suggesting involvement of different types of G-proteins. Recently, G-proteins have also been suggested to couple receptors directly to phopholipase A_2, thus activating arachidonic acid release and prostaglandin production independently of phospholipase C activation, inositol phosphate generation and intracellular Ca^{2+} release [18–22].

The present study was carried out to investigate the

Abbreviations used: G-protein, guanine-nucleotide-binding protein; H7, 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine; PG, prostaglandin; PGI_2, prostacyclin (prostaglandin I_2); TCA, trichloroacetic acid; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; EBSS, Earle's balanced salt solution.

§ To whom correspondence and reprint requests should be addressed, at: Department of Pharmacology, University of Iceland, P.O. Box 8216, 128 Reykjavík, Iceland.

role of G-proteins in endothelial PGI_2 production. We have used the non-specific G-protein activator NaF in combination with $AlCl_3$, presumably acting as AlF_4^-, and have monitored inositol phosphate generation, arachidonic acid release and PGI_2 production. We report on the Ca^{2+}-dependency of arachidonic acid release and on the effects of pertussis toxin and protein kinase C activation or inhibition on inositol phosphate generation, arachidonic acid release and PGI_2 production that follow G-protein activation by AlF_4^-.

MATERIALS AND METHODS

Endothelial cell culture

Endothelial cells were cultured from human umbilical veins as previously described [23]. The cells were harvested by collagenase digestion and seeded on 35 mm culture dishes (Nunc) in medium 199 containing 20% foetal calf serum and antibiotics (penicillin, 100 units/ml and streptomycin, 100 μg/ml). The medium was changed 24 h after seeding the cells and subsequently every 3 days until the cells had reached confluence.

Formation of inositol phosphates

Confluent cells were incubated for 24–36 h in 1 ml of inositol-free medium 199 containing 20% dialysed foetal calf serum, antibiotics and 3 μCi of *myo*-[^{3}H]inositol/ml. After washing the cells twice with Hepes-buffered Earle's balanced salt solution (EBSS), the experiments were performed in 1 ml of this solution containing 20 mM-LiCl. At the time points indicated in each experiment, the medium was removed for measurement of PGI_2 and 1 ml of ice-cold trichloracetic acid (TCA) was added to quench cellular reactions. The inositol phosphates were separated as previously described [1] by applying the samples on columns of anion-exchange resin (AG-1 × 8, 200–400 mesh, formate form) and quantified by liquid scintillation counting.

To permeabilize the cells we used the cell detergent digitonin (10 μg/ml) as described by Muldoon *et al.* [24]. The cells were washed in a cytosolic buffer, consisting of 120 mM-KCl, 10 mM-NaCl, 1 mM-KH_2PO_4, 5 mM-$NaHCO_3$, 10 mM-Hepes, buffered to 150 nM-Ca^{2+} with 0.2 mM-EGTA (calculated according to Thomas [25]). The cells were then incubated in the same buffer containing 10 μg of digitonin/ml for 5 min. The buffer was then removed and the cytosolic buffer containing 100 μM-GTP with or without AlF_4^- (30 mM-NaF/10 μM-$AlCl_3$) was added. At the times indicated, the buffer was removed and 10% ice-cold TCA was added. The inositol phosphates, both in the buffer and in the cells, were then separated.

Prostacyclin production

PGI_2 content was measured in the cell medium at the indicated times by radioimmunoassay for 6-oxo-prostaglandin $F_{1\alpha}$ (6-oxo-$PGF_{1\alpha}$), a stable catabolite of PGI_2, as described previously [1]. The antiserum used is highly specific showing 0.24% cross-reactivity with PGE_1 at 50% displacement (bound/zero bound).

Arachidonic acid release

To measure the release of arachidonic acid, cells were incubated in medium 199 containing 20% foetal calf serum, antibiotics and 1.5 μCi of [^{3}H]arachidonic acid/ml for 24 h. Before experiments, cells were washed twice with Hepes-buffered EBSS, and then 2 ml of the same solution containing fatty-acid free bovine serum albumin (1 mg/ml) was added. To minimize the interference from non-specific release of arachidonic acid that may follow any physical manipulation of cells, the stimulants were not added until 10–20 min later in small volumes of concentrated solutions to give the indicated final concentrations. At the time points indicated, 0.2 ml of the medium was removed and the concentrations of archidonic acid and its labelled metabolites in the sample were quantified by scintillation counting. When the cells were stimulated in low extracellular Ca^{2+}, Hepes-buffered EBSS without Ca^{2+} was used and EGTA (1.0 mM) and $CaCl_2$ (0.30 mM) were added to give the final concentration of 60 nM free Ca^{2+}, calculated according to Thomas [25]. At the times indicated, $CaCl_2$ (1.94 μmol) was added to give 5 μM-free Ca^{2+}.

Materials

myo-[2-^{3}H]Inositol, [^{3}H]inositol phosphate mixture, [^{3}H]arachidonic acid and [^{3}H]6-oxo-$PGF_{1\alpha}$ were obtained from Amersham International; Morgan's medium 199, EBSS and foetal calf serum were obtained from Gibco; collagenase, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H7), NaF, ionophore A23187, digitonin, bovine serum albumin (essentially fatty-acid free), Hepes, pertussis toxin, ATP and LiCl were from Sigma; anion-exchange resin was from Bio-Rad; tissue culture plates were from Flow Laboratories and 6-oxo-$PGF_{1\alpha}$ was from Upjohn. Anti-(6-oxo-$PGF_{1\alpha}$) antiserum was generously supplied by Dr. Michael Dunn, Department of Medicine, Case Western Reserve University, Cleveland, OH, U.S.A.

RESULTS

Production of inositol phosphates and PGI_2 after stimulation with AlF_4^-

Human umbilical vein endothelial cells responded to AlF_4^- by increasing the levels of inositol phosphates in a dose- and time-dependent manner (Fig. 1). When the duration of stimulation was 30 min, peak levels of all inositol phosphates were generated in response to 20–30 mM-NaF in combination with 10 μM-$AlCl_3$ (Fig. 1*a*). The greatest proportional rise was observed in the InsP_2 fraction, but InsP_1 and InsP_3 also rose substantially. At doses above 30 mM there was reduced accumulation of all inositol phosphates.

The levels of the inositol phosphates varied with the duration of incubation with AlF_4^-. When the dose of NaF was 30 mM (Fig. 1*b*), InsP_2 and InsP_3 levels showed the greatest increase between 5 and 10 min and started falling after 30 min. The level of InsP_1 rose throughout the entire experiment. Despite the presence of 20 mM-LiCl to block the degradation of inositol phosphates, their total amount decreased slightly between 30 and 50 min (Fig. 2*b*). When the dose of NaF was increased to 50 mM (Fig. 1*c*), the time–response curve was shifted to the left so that the InsP_2 and InsP_3 had already declined substantially by 30 min, providing a possible explanation for the fall in the total amount of inositol phosphates in the dose–response curve (Figs. 1*a* and 2*a*).

The most striking difference between the AlF_4^--induced accumulation of inositol phosphates and that previously observed after receptor agonist stimulation [1] is the much slower accumulation of inositol phosphates

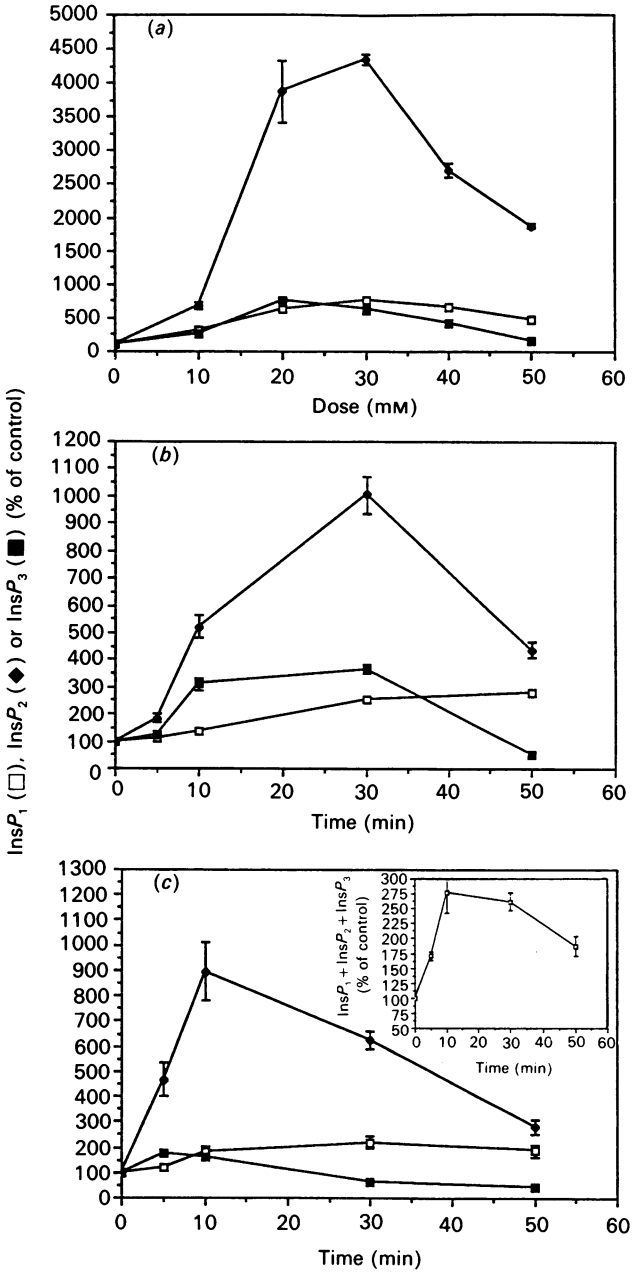

Fig. 1. AlF_4^--induced dose– and time–response curves for the generation of inositol phosphates

[^{3}H]Inositol-labelled endothelial cells were incubated with (*a*) various concentrations of NaF with 10 μM-$AlCl_3$ for 30 min; (*b*) 30 mM-NaF/10 μM-$AlCl_3$ for various periods of time or (*c*) 50 mM-NaF/10 μM-$AlCl_3$ for various periods of time. The inset in (*c*) shows total inositol phosphate generation with time in response to 50 mM-NaF. Data are expressed as radioactivity in inositol phosphates relative to control (100%). Each point is the mean ± S.E.M. (indicated by error bars) of duplicate cultures from a representative of three experiments. The control c.p.m. values for $InsP_1$, $InsP_2$ and $InpP_3$ in (*a*) were 480, 136 and 86, and in (*b*) they were 2258, 557 and 229 respectively.

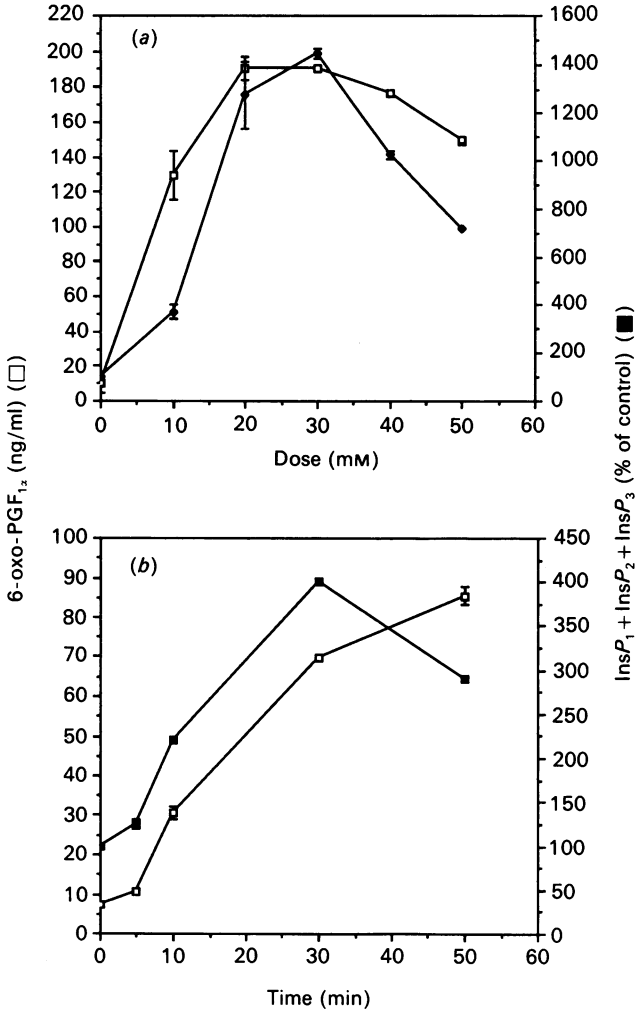

Fig. 2. AlF_4^--induced dose– and time–response curves for the generation of PGI_2 and total inositol phosphates

Prelabelled endothelial cells were incubated with (*a*) various concentrations of NaF with 10 μM-$AlCl_3$ for 30 min, or (*b*) 30 mM-NaF/10 μM-$AlCl_3$ for various lengths of time. Data are expressed as radioactivity in $InsP_1 + InsP_2 + InsP_3$ relative to control (100%) (■), and as ng of 6-oxo-$PGF_{1\alpha}$/ml (□) measured by radioimmunoassay. Each point is the mean ± S.E.M. (indicated by error bars) of duplicate cultures from a representative of three experiments.

due to AlF_4^-. After agonist stimulation, the maximal $InsP_3$ rise was seen within the first 1 min of stimulation [1], but with AlF_4^- the rise did not start appreciably until after 5 min. After permeabilization with digitonin (10 μg/ml), stimulation with AlF_4^- resulted in a faster rise, especially in $InsP_3$, which gave 1.63 and 1.98 times the control value after 1 and 5 min respectively (results not shown), suggesting that slow uptake of AlF_4^- is the main reason for the lag in response.

Inositol phosphate generation has been postulated to play a key role in the regulation of PGI_2 production in endothelial cells. As shown in Figs. 2(*a*) and 2(*b*), treatment with AlF_4^- resulted in a dose- and time-dependent accumulation of 6-oxo-$PGF_{1\alpha}$ with the dose– and time–response curves resembling those for the generation of inositol phosphates.

Ca^{2+}-dependency of AlF_4^--induced arachidonic acid release

To investigate the possibility that arachidonic acid release in endothelial cells might be independent of an

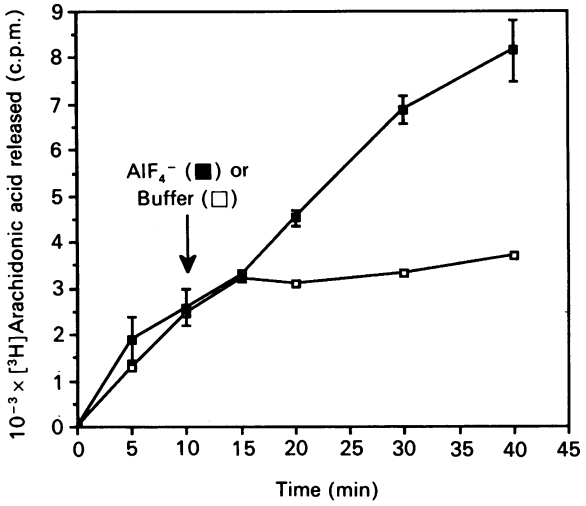

Fig. 3. Time course of AlF_4^--induced arachidonic acid release

[^{3}H]Arachidonic acid-labelled cells were washed twice and incubated in 2 ml of Hepes-buffered EBSS for 10 min, and then buffer (□) or AlF_4^- (■) was added to give the intended final concentration (30 mM-NaF/10 μM-$AlCl_3$). Small aliquots were removed at the times indicated. Data are expressed as accumulated radioactivity in arachidonic acid and its metabolites in the medium. Each point is the mean ± S.E.M. (indicated by error bars) of duplicate cultures from a representative of two experiments.

intracellular Ca^{2+} signal, we examined the intra- and extracellular Ca^{2+} dependency of AlF_4^--induced arachidonic acid release.

AlF_4^- induced a time-dependent release of arachidonic acid (Fig. 3). The time curve is similar to the time curves for inositol phosphate accumulation and PGI_2 production after AlF_4^- stimulation (Fig. 2*b*). To determine the extracellular Ca^{2+} dependency of this arachidonic acid release, the cells were stimulated with AlF_4^- in a solution containing approximately the resting levels of intracellular free Ca^{2+} (50–100 nM) [6,26]. As shown in Fig. 4(*a*), AlF_4^- released arachidonic acid when in a medium with low extracellular Ca^{2+}, but this release came to a halt after about 20 min. When Ca^{2+} was then added to the solution another burst of arachidonic acid release occurred. In order to deplete the intracellular Ca^{2+} stores, the cells were pretreated with the Ca^{2+} ionophore A23187 (2 μM) before AlF_4^- was added (Fig. 4*b*) [6]. This pretreatment totally inhibited the AlF_4^--induced arachidonic acid release when the cells were kept at the low extracellular Ca^{2+} concentration. When Ca^{2+} was then added to the solution, the arachidonic acid release rose rapidly. In Figs. 4(*a*) and 4(*b*), PGI_2 accumulation is indicated below the corresponding curves, demonstrating that the rise in PGI_2 production correlated well with the rise in arachidonic acid release. As shown in Table 1, cells in low extracellular Ca^{2+} were still able to generate inositol phosphates in response to AlF_4^- after ionophore pretreatment.

Pertussis toxin pretreatment

In some cell systems, pertussis toxin discriminates between inositol phosphate formation and arachidonic acid release, suggesting the involvement of distinct G-proteins [21,22]. In order to test this possibility in AlF_4^--stimulated endothelial cells, the cells were pretreated

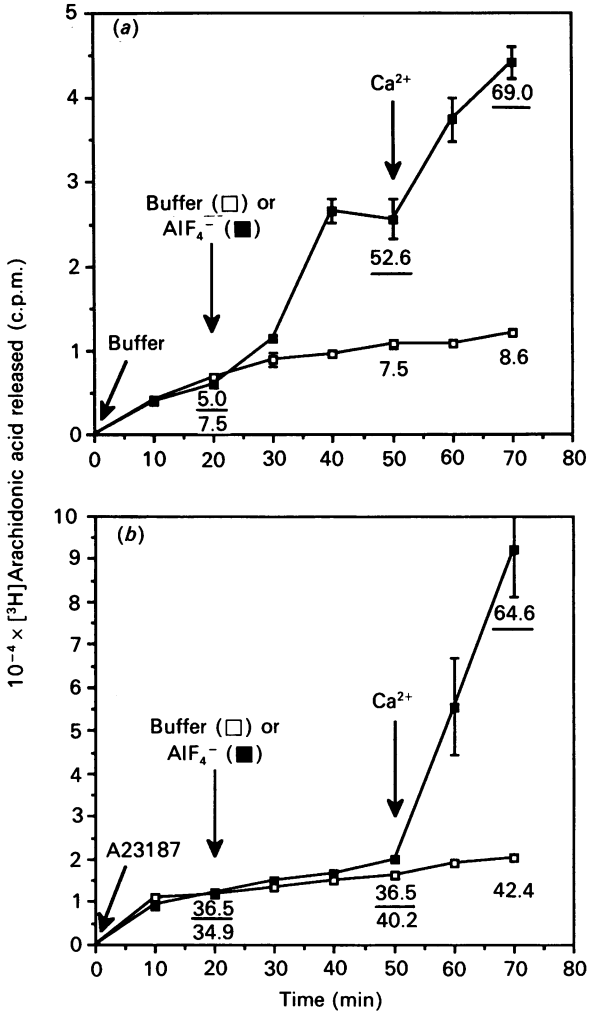

Fig. 4. AlF_4^--induced arachidonic acid release in the presence of low extracellular Ca^{2+} (60 nM) without (*a*) or with (*b*) A23187 pretreatment

Prelabelled cells were incubated in a buffer containing approx. 60 nM free Ca^{2+} with or without the Ca^{2+} ionophore A23187. After 20 min, buffer (□) or AlF_4^- (■) was added to give the intended final concentration (30 mM-NaF/10 μM-$AlCl_3$). Small aliquots were removed at the times indicated. Data are expressed as accumulated radioactivity in arachidonic acid and its metabolites per dish. Each point is the mean ± S.E.M. (indicated by error bars) of duplicate cultures from a representative of three (*a*) or four (*b*) experiments. The numbers below the curves show the concentrations of 6-oxo-$PGF_{1\alpha}$ (ng/culture dish) accumulated at the times indicated. The values for the AlF_4^--treated cells are underlined.

with pertussis toxin (500 ng/ml) for 14 h before stimulation. As shown in Table 2, pertussis toxin affected neither inositol phosphate nor PGI_2 production. In contrast, pretreatment with pertussis toxin inhibited the ATP-stimulated inositol phosphate production by approx. 60% (results not shown), as had previously been reported by Pirotton *et al.* [16], demonstrating activity of pertussis toxin in our cell system.

Role of protein kinase C in the production of PGI_2 and the accumulation of inositol phosphates

In order to investigate the functional role of the diacylglycerol/protein kinase C arm of the phospho-

Table 1. AlF_4^--induced inositol phosphate generation in low extracellular Ca^{2+} (60 nM) with or without A23187 pretreatment

Prelabelled cells were incubated in a buffer containing approx. 60 nM free Ca^{2+} with or without the Ca^{2+} ionophore A23187. After 20 min, buffer or AlF_4^- was added to give the intended final concentration (30 mM-NaF/10 μM-$AlCl_3$), and 30 min later the medium was removed and TCA was added. Data are expressed as radioactivity in $InsP_1 + InsP_2 + InsP_3$ relative to control (100%). Each value is the mean ± S.E.M. of duplicate cultures from a representative of two experiments.

Pretreatment	Treatment	$InsP_1 + InsP_2 + InsP_3$ (% of control)
Buffer	Buffer	100.0 ± 2.5
Buffer	AlF_4^-	282.5 ± 5.8
A23187	Buffer	121.8 ± 10.1
A23187	AlF_4^-	268.0 ± 22.0

inositide system, we studied the effects of TPA, a protein kinase C activator and of the protein kinase C inhibitor H7 on the accumulation of inositol phosphates and the production of PGI_2.

TPA treatment for 5 min before adding AlF_4^- resulted in decreased accumulation of inositol phosphates (Fig. 5). After 20 min of stimulation, the TPA-mediated inhibition was 47% and had reached 71% after 30 min of stimulation. Conversely, pretreatment with H7, a protein kinase C inhibitor, enhanced the generation of inositol phosphates after AlF_4^- stimulation by 28% (Table 3).

For comparison, the effects of TPA and H7 on the production of PGI_2 after AlF_4^- stimulation were examined. As shown in Fig. 6, TPA markedly stimulated the production of PGI_2. After TPA pretreatment, PGI_2 production occurred at a lower concentration of AlF_4^- (Fig. 6*a*) and also after a shorter time of stimulation (Fig. 6*b*). TPA alone for 35 min did not induce PGI_2 production (Fig. 6*a*). Conversely, H7 reduced PGI_2 production both after TPA pretreatment and also after AlF_4^- stimulation alone (Table 4). The H7-induced inhibition was 29% after AlF_4^- stimulation alone and 38% when the cells had been pretreated with TPA. Finally, in order to further localize the effects of protein kinase C stimulation of PGI_2 production, we examined the effect of TPA pretreatment on AlF_4^--induced arachidonic acid release. As shown in Fig. 7, TPA both increased the amount of arachidonic acid released and reduced the time needed for the release to occur after stimulation by AlF_4^-.

DISCUSSION

This study shows that human umbilical vein endothelial cells exposed to the G-protein activator AlF_4^- respond by increasing the level of inositol phosphates, releasing arachidonic acid and producing PGI_2. We also report on the involvement of Ca^{2+} and protein kinase C in these processes and examine the effects of pertussis toxin pretreatment on the response of endothelial cells to AlF_4^-.

Although still not specifically identified, G-proteins have been widely suggested to couple receptors to phospholipase C [10]. These suggestions are based on experiments using the non-specific G-protein activators AlF_4^- [13] and non-hydrolysable GTP analogues [12,13] and the more specific modulator of G-proteins, pertussis toxin. Sensitivity to cholera toxin and pertussis toxin has been used to distinguish between different types of G-proteins. Judging from pertussis toxin sensitivity, the type of G-protein involved in the activation of phospholipase C seems to vary between different cell types [14,15] and even between different receptors on the same cell type [16,17]. Of the two non-specific G-protein activator groups, AlF_4^- has the advantage over GTP analogues of not requiring permeabilization of the cells. This is important in our system because of the concurrent examination of arachidonic acid release and PGI_2 production.

The effects of dose and time on inositol phosphate generation, arachidonic acid release and PGI_2 production in response to AlF_4^- were very similar (Figs. 2 and 3), suggesting a causal relationship. The slow accumulation of both the inositol phosphates and PGI_2 compared with receptor agonists is most probably due to a different mechanism of activation. Whereas the receptor agonists have their effect on the receptor outside the cell, AlF_4^- has its effect on the G-proteins and must thus be taken up by the cell.

As shown in Fig. 2(*b*), the total amount of inositol phosphates fell after 30 min despite the LiCl (20 mM) used to block degradation. This decrease is probably due to incomplete blocking by LiCl. The time response for 50 mM-NaF (Fig. 1*c*) was shifted to the left compared with that for 30 mM (Fig. 1*b*), with the total amount of inositol phosphates falling after 10 min of stimulation.

Table 2. Effects of pertussis toxin pretreatment on AlF_4^--stimulated inositol phosphate and PGI_2 generation

Prelabelled endothelial cells were pretreated with or without pertussis toxin (500 ng/ml) for 14 h and then incubated for 30 min in buffer or AlF_4^- (30 mM-NaF/10 μM-$AlCl_3$). Data are expressed as radioactivity in $InsP_1 + InsP_2 + InsP_3$ relative to the control (100%), and as ng of 6-oxo-$PGF_{1\alpha}$/ml measured by radioimmunoassay. Each value is the mean ± S.E.M. of duplicate cultures from a representative of two experiments.

Pretreatment	Treatment	$InsP_1 + InsP_2 + InsP_3$ (% of control)	6-Oxo-$PGF_{1\alpha}$ (ng/ml)
Buffer	Buffer	100.0 ± 3.9	4.2 ± 1.8
Pertussis toxin	Buffer	98.0 ± 1.5	5.3 ± 1.2
Buffer	AlF_4^-	202.0 ± 1.2	91.2 ± 3.0
Pertussis toxin	AlF_4^-	200.7 ± 8.2	108.1 ± 3.6

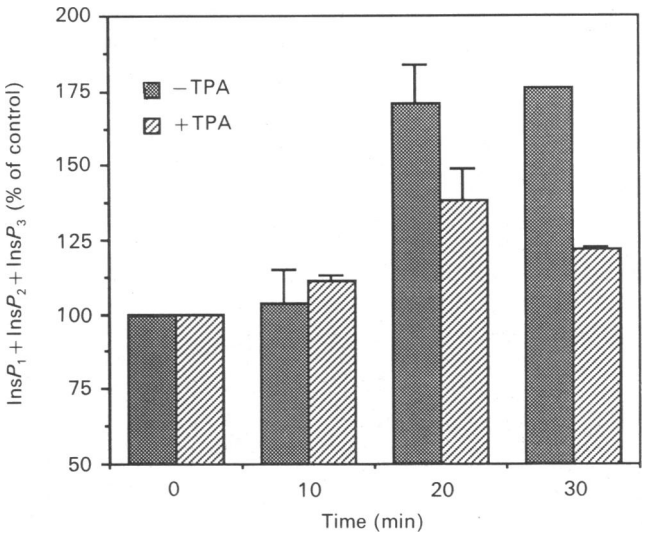

Fig. 5. The effects of TPA on AlF_4^--induced inositol phosphate generation

Prelabelled cells were incubated with or without TPA (100 ng/ml) for 5 min. AlF_4^- (30 mM-NaF/10 μM-$AlCl_3$) was then added for the times indicated. Data are expressed as radioactivity in $InsP_1 + InsP_2 + InsP_3$ relative to the control (100%). Each bar represents the mean ± S.E.M. (indicated by error bars) of duplicate cultures from a representative of two experiments.

Consequently, in the combined dose–response curves depicted in Fig. 2(*a*) representing 30 min of stimulation, there is a significant fall in total inositol phosphates at 50 mM. Since the PGI_2 curve represents accumulation of a stable catabolite, no such fall would be expected. The similarity in the dose– and time–response curves for the inositol phosphate and PGI_2 production after direct G-protein activation by AlF_4^- suggests a causal relationship and mimics the response to receptor activation of endothelial cells with the agonists bradykinin, thrombin and histamine [1–3]. These receptor agonists also increase the intracellular Ca^{2+} levels [2,6,26], and Hallam *et al.* [6] have provided strong evidence that this increase in intracellular Ca^{2+} is a determining factor in PGI_2 production after thrombin activation. Their results argue quite strongly for a causal relationship between receptor-agonist-stimulated inositol phosphate generation and PGI_2 production in endothelial cells stimulated with thrombin. However, recently there have been indications that arachidonic acid might be released from cell membranes independently of inositol phosphate generation, possibly due to direct coupling of a receptor to phospholipase A_2 by a G-protein [18–22]. In endothelial cells also there are some indications that arachidonic acid might be released independently of inositol phosphate generation. Pertussis toxin has been shown to inhibit inositol phosphate generation after ATP stimulation, while stimulating the production of PGI_2 [16]. Leukotriene D_4, which

Table 3. Effects of H7 pretreatment on AlF_4^--induced inositol phosphate generation

Prelabelled endothelial cells were incubated in a buffer with or without H7 (100 μM) for 10 min. Buffer or AlF_4^- (30 mM-NaF/10 μM-$AlCl_3$) was then added for 30 min. Data are expressed as radioactivity in $InsP_1 + InsP_2 + InsP_3$ relative to the control. Each value is the mean ± S.E.M. of duplicate cultures from a representative of two experiments.

Pretreatment	Treatment	$InsP_1 + InsP_2 + InsP_3$ (% of control)
Buffer	Buffer	100 ± 2
H7	Buffer	104 ± 9
Buffer	AlF_4^-	262 ± 18
H7	Alf_4^-	312 ± 13

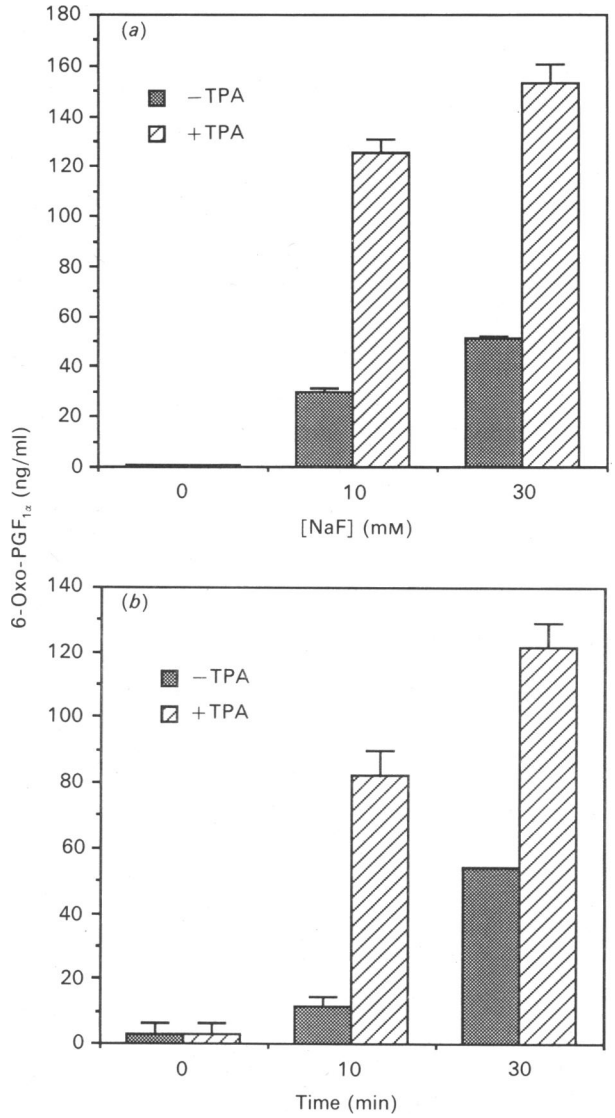

Fig. 6. The effects of TPA on AlF_4^--induced PGI_2 production

Prelabelled cells were incubated with or without TPA (100 ng/ml) for 5 min, and then (*a*) various concentrations of NaF plus 10 μM-$AlCl_3$ for 30 min, or (*b*) AlF_4^- (30 mM-NaF/10 μM-$AlCl_3$) for the times indicated were added. Data are expressed as ng of 6-oxo-$PGF_{1\alpha}$ produced/ml measured by radioimmunoassay. Each bar represents the mean ± S.E.M. (indicated by error bars) of duplicate cultures from a representative of three experiments.

Table 4. Effects of H7 on AlF_4^--stimulated or AlF_4^--plus-TPA-stimulated PGI_2 production

Endothelial cells were washed twice with or without H7 and then incubated in a buffer with or without H7 (100 μM) and/or TPA (100 ng/ml). After 10 min, buffer or AlF_4^- (30 mM-NaF/10 μM-$AlCl_3$) was added for 30 min. Data are expressed as ng of 6-oxo-$PGF_{1\alpha}$/ml measured by radioimmunoassay. Each value is the mean ± S.E.M. of duplicate cultures from a representative of two experiments.

Pretreatment	Treatment	6-Oxo-$PGF_{1\alpha}$ (ng/ml) −H7	+H7
Buffer	Buffer	5.8±0.1	3.5±1.5
TPA	Buffer	6.4±1.4	3.3±0.1
Buffer	AlF_4^-	78.4±2.4	54.8±12.2
TPA	AlF_4^-	114.8±0.2	70.5±1.6

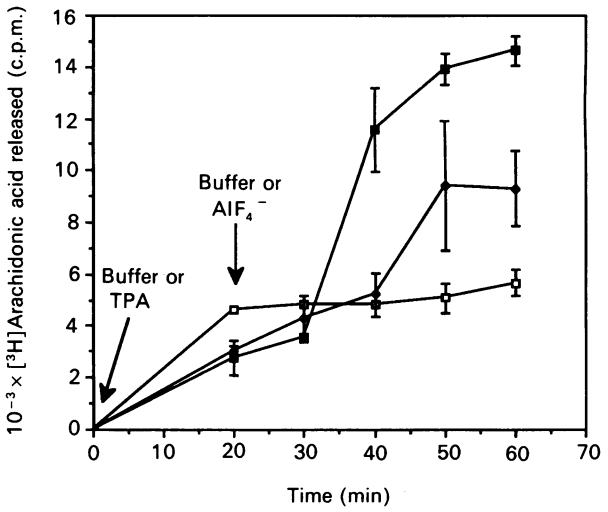

Fig. 7. The effects of TPA on AlF_4^--induced arachidonic acid release

[^{3}H]Arachidonic acid-labelled cells were incubated with or without TPA for 20 min and buffer or AlF_4^- was then added to give the intended final concentration (30 mM-NaF/10 μM-$AlCl_3$). Small aliquots were removed at the times indicated. Data are expressed as accumulated radioactivity in arachidonic acid and its metabolites per dish. Each point is the mean ± S.E.M. (indicated by error bars) of duplicate cultures from a representative of two experiments. □, Buffer alone; ◆, AlF_4^-; ■, AlF_4^-+TPA.

induces PGI_2 production in endothelial cells [27], has been reported to activate phospholipase A_2 without affecting the activity of phospholipase C in membrane preparations from endothelial cells [28]. In view of these results, the possibility has to be considered that AlF_4^--induced arachidonic acid release is not mediated by inositol phosphate generation but by a direct G-protein activation of phospholipase A_2. Such activation could be either the result of a G-protein-coupled opening of a Ca^{2+} channel, and thus be dependent on extracellular Ca^{2+}, or a direct activation of phospholipase A_2 independent of increased cytoplasmic Ca^{2+} levels. In order to investigate these possibilities, we examined the intra- and extracellular Ca^{2+} dependency of the arachidonic acid release after AlF_4^- stimulation. When the cells were stimulated with AlF_4^- in a medium containing a low Ca^{2+} concentration (60 nM), selected to approximate to resting intracellular Ca^{2+} levels, the cells were still able to release arachidonic acid. The arachidonic acid release came to a halt after 20 min but another burst of arachidonic acid release was detected when Ca^{2+} was added to the buffer (Fig. 4*a*). The first phase of the thrombin-activated Ca^{2+} rise has been reported to be independent of extracellular Ca^{2+}[29]. These results argue against the possibility that AlF_4^--stimulated arachidonic acid release is solely dependent on extracellular Ca^{2+}. To discriminate between arachidonic acid release mediated by a Ca^{2+} signal from intracellular Ca^{2+} stores and a direct activation of phospholipase A_2, the cells were again maintained in the very low extracellular Ca^{2+}, but before AlF_4^- stimulation they were pretreated with the Ca^{2+} ionophore A23187 which has been shown to deplete releasable intracellular Ca^{2+} stores in endothelial cells [6]. As shown in Fig. 4(*b*), this pretreatment totally inhibited AlF_4^--induced arachidonic acid release. However, when Ca^{2+} was added to the medium there was a rapid release of arachidonic acid. PGI_2 production correlated well with the arachidonic acid release in these experiments (Figs. 4*a* and 4*b*). The ionophore pretreatment in low extracellular Ca^{2+} did not inhibit the generation of inositol phosphates (Table 1), excluding the possibility that this treatment was preventing the activation of phospholipase C. Emptying releasable intracellular Ca^{2+} stores thus inhibits AlF_4^--induced arachidonic acid release, suggesting that this release is dependent on intracellular Ca^{2+} stores and thus on activation of the phosphoinositide second messenger system. This conclusion is supported by the similar dose- and time-response curves for the increases in inositol phosphate accumulation, arachidonic acid release and PGI_2 production. The lack of effect of pertussis toxin on the production of both inositol phosphates and PGI_2 after AlF_4^- stimulation (Table 2) is also consistent with this conclusion.

We have previously shown that the protein kinase C activator TPA inhibits the generation of inositol phosphates after receptor activation with thrombin [1]. TPA also inhibits the receptor-activated Ca^{2+} rise in endothelial cells [8]. This suggests that protein kinase C activation has a negative feedback role in endothelial cells, in accordance with the general feedback concept [4]. When AlF_4^--stimulated cells were pretreated with TPA, the generation of inositol phosphates was also markedly inhibited (Fig. 5). Since AlF_4^- has its effect distal to the receptor in the cascade of signal transduction, these results suggest that TPA has its inhibitory effect at the level of a G-protein or possibly phospholipase C. Similar results have been obtained in fibroblasts [30] and hepatocytes [31] after AlF_4^- stimulation. However, the TPA-activated protein kinase C arm has more than only a negative feedback role in cells. It has been shown both to induce arachidonic acid release by itself [32] and also to enhance arachidonic acid release in response to increased Ca^{2+} levels [33,34]. Work in our laboratory has demonstrated that the effect of TPA pretreatment on PGI_2 production in endothelial cells after thrombin activation was dependent on both the concentration of

thrombin and the duration of TPA pretreatment [1]. These results suggested a separate effect of protein kinase C on PGI_2 production, independent of the effects on inositol phosphate generation. In the present study, pretreatment with TPA greatly amplified AlF_4^--induced PGI_2 production, in contrast with the inhibition of inositol phosphate generation, although TPA by itself did not induce PGI_2 production (Figs. 6*a* and 6*b*). The effects of H7 on the endothelial cell response to AlF_4^- were opposite to the effects of TPA, with respect to both inositol phosphate generation and PGI_2 production (Tables 3 and 4). The results showing TPA-enhanced arachidonic acid release in response to AlF_4^- (Fig. 7) suggest that protein kinase C, when activated by TPA, stimulates PGI_2 production by stimulating phospholipase A_2 directly, or possibly through a regulatory protein. The demonstration that TPA inhibits inositol phosphate generation while potentiating PGI_2 production might seem contrary to the proposition that the phosphoinositide second messenger system is the key regulator for the PGI_2 production. But as the TPA-induced inhibition of inositol phosphate generation and Ca^{2+} release is only partial [1,8] and as TPA also has a stimulatory effect on arachidonic acid release, the outcome is dependent on which effect (inhibitory or stimulatory) is dominant, and thus does not exclude a causal relationship between the two events.

Although TPA-activated protein kinase C thus seems to have a complex effect on the production of PGI_2, there are reports that argue against a role for protein kinase C in this signal transduction pathway after agonist stimulation. Demolle *et al.* [35] have observed that the phosphorylation pattern after receptor agonist activation of endothelial cells has more resemblance to the pattern seen after Ca^{2+} ionophore treatment than the pattern observed after TPA treatment, suggesting that Ca^{2+}/calmodulin kinase rather than protein kinase C is activated. Hallam *et al.* [6] compared thrombin- and Ca^{2+} ionophore-induced PGI_2 production and suggested that the major (and probably exclusive) intracellular stimulus for the production of PGI_2 in response to thrombin is the elevation of intracellular Ca^{2+}. A similar suggestion was made after comparing ATP- and Ca^{2+} ionophore-stimulated PGI_2 production [36]. Those findings thus suggest that the $InsP_3$ arm of the phosphoinositide system in endothelial cells, resulting in an elevation in intracellular Ca^{2+} is the major mediator of signal transduction, although diacylglycerol has been shown to be formed in these cells after agonist stimulation [7]. In contrast with these findings. Whatley *et al.* [37] have recently reported that protein kinase C has a stimulatory role in arachidonic acid release and platelet activating factor formation in endothelial cells, but only in the presence of a stimulus that increases intracellular Ca^{2+}. These findings are consistent with our observation that TPA stimulates arachidonic acid release and PGI_2 production only in the presence of AlF_4^- stimulation but not by itself.

In summary, we have demonstrated that stimulation of human umbilical vein endothelial cells with the non-specific G-protein activator AlF_4^- resulted in a rise of inositol phosphates, a release of arachidonic acid and the production of PGI_2. This stimulation was pertussis toxin-insensitive. The phosphoinositide second messenger system seems to be the main regulator of arachidonic acid release and thus PGI_2 production since: (*a*) AlF_4^- could not release arachidonic acid in low extracellular Ca^{2+} if intracellular Ca^{2+} stores were depleted, suggesting that arachidonic acid release is dependent on intracellular Ca^{2+} stores, and (*b*) there was close resemblance between the dose- and time–response curves for the three events; inositol phosphate generation, arachidonic acid release and PGI_2 production. Finally, we report further evidence that protein kinase C may have a complex regulatory role in the production of PGI_2, affecting inositol phosphate generation and the release of arachidonic acid in opposite directions.

We thank Ásrún Kristmundsdóttir and Arndís Theodors for skilled technical assistance. This work was supported in part by a grant from the Icelandic Science Fund. M.K.M. was the recipient of a grant from the H. Jónsdóttir and S. Kristjánsson Memorial Fund.

REFERENCES

1. Halldórsson, H., Kjeld, M. & Thorgeirsson, G. (1988) Arteriosclerosis **8**, 147–154
2. Jaffe, E. A., Grulich, J., Weksler, B. B., Hampel, G. & Watanabe, K. (1987) J. Biol. Chem. **262**, 8557–8565
3. Derian, C. K. & Moskowitz, M. A. (1986) J. Biol. Chem. **261**, 3831–3837
4. Berridge, M. J. (1987) Annu. Rev. Biochem. **56**, 159–193
5. Nishizuka, Y. (1986) Science **233**, 305–312
6. Hallam, T. J., Pearson, J. D. & Needham, L. A. (1988) Biochem. J. **251**, 243–249
7. Moscat, J., Moreno, F. & García-Barreno, P. (1987) Biochem. Biophys. Res. Commun. **145**, 1302–1309
8. Brock, T. A. & Capasso, E. L. (1988) J. Cell. Physiol. **136**, 54–62
9. Gilman, A. G. (1987) Annu. Rev. Biochem. **56**, 615–649
10. Cockcroft, S. (1987) Trends Biochem. Sci. **12**, 75–78
11. Litosch, I., Wallis, C. & Fain, J. (1985) J. Biol. Chem. **260**, 5464–5471
12. Cockcroft, S. & Gomperts, B. D. (1985) Nature (London) **314**, 534–536
13. Blackmore, P. F., Bocckino, S. B., Waynick, L. E. & Exton, J. E. (1985) J. Biol. Chem. **260**, 14477–14483
14. Ohta, H., Okajima, F. & Ui, M. (1985) J. Biol. Chem. **260**, 15771–15780
15. Masters, S. B., Martin, M. W., Harden, T. K. & Brown, T. K. (1985) Biochem. J. **227**, 933–937
16. Pirotton, S., Erneux, C. & Boeynaems, J. M. (1987) Biochem. Biophys. Res. Commun. **147**, 1113–1120
17. Lambert, T. L., Kent, R. S. & Whorton, A. R. (1986) J. Biol. Chem. **261**, 15288–15293
18. Slivka, S. R. & Insel, P. A. (1987) J. Biol. Chem. **262**, 4200–4207
19. Slivka, S. R. & Insel, P. A. (1988) J. Biol. Chem. **263**, 14640–14647
20. Burch, R. M. & Axelrod, J. (1987) Proc. Natl. Acad. Sci. U.S.A. **84**, 6374–6378
21. Burch, R. M., Luini, A. & Axelrod, J. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 7201–7205
22. Fuse, I. & Tai, H. H. (1987) Biochem. Biophys. Res. Commun. **146**, 659–665
23. Thorgeirsson, G., Robertson, A. L., Jr. & Cowan, D. H. (1979) Lab. Invest. **41**, 51–62
24. Muldoon, L. L., Jamieson, G. A. & Villeral, M. L. (1987) J. Cell. Physiol. **130**, 29–36
25. Thomas, M. V. (1982) Techniques in Calcium Research (Biological Techniques Series) pp. 40–47, Academic Press, London and New York

26. Rotrosen, D. & Gallin, J. I. (1986) J. Cell Biol. **103**, 2379–2387
27. Clark, M. A., Littlejohn, D., Mong, S. & Crooke, S. T. (1986) Prostaglandins **31**, 157–166
28. Clark, M. A., Littlejohn, D., Conway, T. M., Mong, S., Steiner, S. & Crooke, S. T. (1986) J. Biol. Chem. **261**, 10713–10718
29. Hallam, T. J., Jacob, R. & Merritt, J. E. (1988) Biochem. J. **255**, 179–184
30. Paris, S. & Pouysségur, J. (1987) J. Biol. Chem. **262**, 1970–1976
31. Blackmore, P. F. & Exton, J. H. (1986) J. Biol. Chem. **261**, 11056–11063
32. Parker, J., Daniel, L. W. & Waite, M. (1987) J. Biol. Chem. **262**, 5385–5393
33. Halenda, S. P. & Rehm, A. G. (1987) Biochem. J. **248**, 471–475
34. Bonventre, J. V. & Swidler, M. (1988) J. Clin. Invest. **82**, 168–176
35. Demolle, D., Lecomte, M. & Boeynaems, J. M. (1988) J. Biol. Chem. **263**, 18459–18465
36. Carter, T. D., Hallam, T. J., Cusack, N. J. & Pearson, J. D. (1988) Br. J. Pharmacol. **95**, 1181–1190
37. Whatley, R. E., Nelson, P., Zimmerman, G. A., Stevens, D. L., Parker, C. J., McIntyre, T. M. & Prescott, S. M. (1989) J. Biol. Chem. **264**, 6325–6333

Received 24 February 1989/6 July 1989; accepted 17 July 1989