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Protein kinase C ε-dependent pathway of extracellular signal-regulated protein kinase activation by $P2Y_1$ and $P2Y_2$ purinoceptors that activate cytosolic phospholipase A_2 in endothelial cells

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Abstract

The aim of this study was to investigate the stimulating effects on arachidonic acid release of $P2Y_1$ and $P2Y_2$ receptor-selective agonists, 2-methylthio-ATP (2MeSATP) and UTP, respectively, in bovine pulmonary artery endothelial cells. Exposure of cells to 2MeSATP and UTP led to the release of arachidonic acid, a response which was abolished by the removal of extracellular Ca^{2+} and methyl arachidonyl fluorophosphonate. Phorbol 12-myristate 13-acetate (PMA) itself not only stimulated arachidonic acid release but also played a permissive role in the response to UTP. However, PMA failed to enhance the arachidonic acid response induced by 2MeSATP, probably due to greater attenuation of the $[Ca^{2+}]_i$ increase caused by 2MeSATP than UTP. Inhibition of protein kinase C with Ro 31-8220 (1-[3-(amidinothio) propyl-1*H*-indoyl-3-yl]-3-(1-methyl-1*H*-indoyl-3-yl)-maleimide-methane sulphate) and staurosporine, but not with Go 6976 (12-(-2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-indolo(2,3-a)pyrrolo(3,4-c)carbazole), reduced the arachidonic acid response of 2MeSATP, UTP and PMA. PMA-induced potentiation of the UTP response reached a maximum after a 1-h preincubation, then declined and eventually lost its effect when the preincubation lasted up to 8 h. Among the protein kinase C isoforms present in endothelial cells, βI and ε could be down-regulated by treatment with PMA for 4–24 h. PD 098059 (2-(2-Amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one) inhibited extracellular signal-regulated protein kinase activation, cytosolic phospholipase A_2 phosphorylation and arachidonic acid release caused by 2MeSATP, UTP and PMA. Taken together, our results demonstrate that $P2Y_1$ and $P2Y_2$ purinoceptors mediate arachidonic acid release by activating cytosolic phospholipase A_2 through an elevation of $[Ca^{2+}]_i$ and protein kinase C ε-, extracellular signal-regulated protein kinase-dependent phosphorylation.

Keywords: Protein kinase C; Ca^{2+}; Phospholipase A_2; Cytosolic; P2Y purinoceptor subtype; Protein kinase, extracellular signal-regulated

1. Introduction

Phospholipase A_2 plays a central role in the production of inflammatory mediators, the eicosanoids and platelet-activating factor. Mammalian cells contain multiple structurally diverse forms of phospholipase A_2, and there has been considerable interest in determining the role of a specific phospholipase A_2 in mediating arachidonic acid release. Phospholipase A_2s have been divided into several groups based on molecular weight. Groups I, II and III are small Ca^{2+}-dependent secretory phospholipase A_2s, and group IV is an intracellular cytosolic, 85-kDa Ca^{2+}-dependent phospholipase A_2 (Mayer and Marshall, 1993; Dennis, 1997; Leslie, 1997). It has become clear that phospholipase A_2 represents a growing superfamily of enzymes, since many newly identified phospholipase A_2s have been reported (Dennis, 1997; Leslie, 1997).

Many recent studies have shown that cytosolic phospholipase A_2 contains multiple phosphorylation sites on Ser/Thr. Importantly, the catalytic activity of cytosolic phospholipase A_2 is increased after the phosphorylation of Ser^{505} by p42 extracellular signal-regulated protein kinase (Lin et al., 1992; Lin et al., 1993; Nemenoff et al., 1993). Moreover, it has been shown that in response to the increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$), cytosolic phospholipase A_2 is translocated from the cytosol to the cell membrane, e.g., nuclear envelope and endoplasmic reticulum. This process is mediated by the Ca^{2+}-dependent phospholipid-binding domain located in the N-terminal

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region of cytosolic phospholipase A_2 (Channon and Leslie, 1990; Clark et al., 1991; Paglin et al., 1993; Schievella et al., 1995). It is believed that both Ca^{2+} and phosphorylation of Ser^{505} mediate the rapid activation of cytosolic phospholipase A_2 upon hormone stimulation.

Eicosanoids elicit multiple physiologic and pathophysiologic responses in the vascular system. For example, prostacyclin is an important autacoid regulating vascular tone and platelet aggregation (Moncada et al., 1976). Arachidonic acid is necessary for growth factor-stimulated endothelial cell movement (Sa and Fox, 1994) and proliferation (Handler et al., 1990). Besides, another phospholipase A_2 metabolite, lysophosphatidylcholine, is also known to be a potent monocyte chemoattractant and participates in the initial step in the development of atherosclerotic lesions (Quinn et al., 1988).

Based on the pivotal role of eicosanoids in the vascular system, it is interesting to understand the physiological factors which are able to stimulate arachidonic acid release and the mechanism involved. Previous studies have shown that endothelial cells express two purinoceptors, $P2Y_1$ and $P2Y_2$, and that extracellular ATP, via activation of purinoceptor-coupled phospholipase C, can cause the release of prostacyclin (Lustig et al., 1992; Boarder et al., 1995; Patel et al., 1996a). Studies of several nucleotide analogues acting on P2Y purinoceptors also indicate that the agonist potency profiles are 2-methylthio-ATP (2MeSATP) > ATPγS > ATP ≥ ADP ≫ α,β-methylene ATP, UTP for $P2Y_1$ receptors, and UTP = ATP ≥ ATPγS ≫ ADP, 2MeSATP, α,β-methylene ATP for $P2Y_2$ receptors (King et al., 1998). Moreover, while phosphorylation and activation of p42 and p44 extracellular signal-regulated protein kinases are involved in the stimulation of endothelial prostacyclin production by P_2 purinoceptors (Patel et al., 1996b), the direct evidence for cytosolic phospholipase A_2 activation and the detailed signal mechanism triggered by these two P2Y receptor subtypes have not been shown. In bovine pulmonary artery endothelial cells, we have demonstrated the presence of $P2Y_1$ and $P2Y_2$ purinoceptors, both of which are coupled to the activation of phosphoinositide-specific phospholipase C via a pertussis toxin-insensitive G protein (Chen et al., 1996). Here, this study was carried out to determine the downstream mechanism associated with cytosolic phospholipase A_2 activation by $P2Y_1$ and $P2Y_2$ receptor-selective agonists. The roles of $[Ca^{2+}]_i$, protein kinase C, and extracellular signal-regulated protein kinase will be discussed.

2. Materials and methods

2.1. Reagents

Minimun essential medium (MEM) and fetal bovine serum were obtained from Gibco BRL (Grand Island, NY). [^{3}H]arachidonic acid (100 Ci/mmol) and [γ-^{32}P]ATP (5000 Ci/mmol) were purchased from New England Nuclear (Boston, MA). Horseradish peroxidase-conjugated goat anti-mouse and sheep anti-rabbit antibodies were purchased from Amersham Pharmacia Biotech. Staurosporine, phorbol 12-myristate 13-acetate (PMA), ionomycin, thapsigargin, UTP, ATP, α,β-methylene ATP, adenosine, myelin basic protein, and {1-[2-(5-Carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2′-amino-5′-methylphenoxy)-ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid pentaacetoxymethyl ester} (Fura-II/AM) were obtained from Sigma (St. Louis, MO). PD 098059 (2-(2-Amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one) and 2MeSATP were from RBI (Natick, MA). Ro 31-8220 (1-[3-(amidinothio) propyl-1*H*-indoyl-3-yl]-3-(1-methyl-1*H*-indoyl-3-yl)-maleimide-methane sulphate) and Go 6976 (12-(-2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-indolo-(2,3-a)pyrrolo(3,4-c)carbazole) were purchased from Calbiochem (La Jolla, CA). Methyl arachidonyl fluorophosphonate was purchased from Cayman (Ann Arbor, MI). Monoclonal antibodies to protein kinase C α, γ, δ, ε, θ, λ, μ, ι, ζ and extracellular signal-regulated protein kinase were purchased from Transduction Laboratories (Lexington, KY), and polyclonal antibodies to protein kinase C βI, βII and η were from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody specific to phosphorylated extracellular signal-regulated protein kinases was obtained from New England BioLabs (Beverly, MA). RHC 80267 (1,6-bis-(cyclohexyloximinocarbonylamino)-hexane) was purchased from Biomol (Plymouth Meeting, PA). All the materials for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were obtained from Bio-Rad Laboratories (Hercules, CA).

2.2. Cell culture

Bovine pulmonary artery endothelial cells obtained from the American Type Culture Collection were grown as monolayer cultures in MEM supplemented with 10% fetal bovine serum at 37°C, in a humidified atmosphere of 95% air/5% CO_2.

2.3. [^{3}H]arachidonic acid release

Arachidonic acid release was measured as described before (Lin and Lee, 1996). Briefly, confluent cells grown in 24-well plates (approximately $8–10 \times 10^4$ cells/well) were prelabeled with 0.3 μCi/ml of [^{3}H]arachidonic acid in MEM for 24 h at 37°C. The cells were washed three times with serum-free MEM and incubated in medium containing 0.5% fatty acid-free bovine serum albumin before stimulation with 2MeSATP, UTP, ionomycin, thapsigargin or PMA at 37°C for 30 min. At the end of the incubation, the medium was removed and centrifuged at $250 \times g$ for 5 min to remove floating cells. The radioactivity in the supernatant was then measured.

2.4. Western blotting and mobility shift assay of $cPLA_2$

Bovine pulmonary artery endothelial cells treated with stimuli as indicated were washed three times with ice-cold phosphate-buffered saline (PBS) and the plates were immediately placed on ice to stop the reactions. The cells were scraped from the plates in lysis buffer (20 mM Tris–HCl, 0.5 mM EGTA, 2 mM EDTA, 2 mM dithiothreitol, 0.5 mM *p*-methylsulfonyl fluoride, 10 μg/ml leupeptin, pH 7.5), transferred to microfuge tubes and sonicated. The protein levels in cell lysates were assayed by the Bradford method. Equal amounts (50–80 μg) of cell lysates were treated with 5× Laemmli buffer, boiled for 5 min and subjected to 9% (for protein kinase C) or 10% (for cytosolic phospholipase A_2) SDS–PAGE. The resolved proteins were transferred to nitrocellulose membranes (Amersham Pharmacia Biotech ECL grade) and blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween-20 (TBST) overnight at 4°C. After three washes with TBST, the membrane was incubated with protein kinase C isoform-specific primary antibody, phosphorylated extracellular signal-regulated protein kinase antibody, or cytosolic phospholipase A_2 antibody, each diluted in TBST, for 90 min. After further washes, the immunoreactive bands were detected with the enhanced chemiluminescence system (Amersham Pharmacia Biotech) following incubation with horseradish peroxidase-conjugated immunoglobulin G and the substrate luminol.

2.5. Immunoprecipitation and extracellular signal-regulated protein kinase assay

After the cells were starved for 2 days, the cell extracts in lysis buffer (20 mM Tris, pH 7.5, 1 mM $MgCl_2$, 125 mM NaCl, 1% Triton X-100, 1 mM *p*-methylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 50 mM NaF, 25 mM β-glycerophosphate, 100 μM vanadate) were immunoprecipitated by incubation overnight with anti-extracellular signal-regulated protein kinase antibody and protein A/G-Sepharose beads at 4°C. The beads were washed three times with 1 ml of ice-cold whole cell extract buffer. The immune-complex kinase assay of immunoprecipitates was performed at 30°C for 30 min in 20 μl of kinase reaction buffer (25 mM HEPES, pH 7.4, 20 mM $MgCl_2$, 0.1 mM Na_3VO_4, 2 mM dithiothreitol, 50 μg/ml myelin basic protein, 25 μM ATP, 3 μCi [γ-^{32}P]ATP). The reaction was terminated with 5× Laemmli sample buffer, and the products were resolved by 15% SDS–PAGE gel electrophoresis. The phosphorylated myelin basic protein was visualized by autoradiography. The results were quantitated by Phosphorimager (Molecular Dynamics) analysis.

2.6. $[Ca^{2+}]_i$ measurement

Cells grown on 24-mm glass slides were incubated with Fura-II/AM (3 μM) and pluronic F-127 (0.02% v/v) in MEM at 37°C for 60 min. Cells were washed twice with physiological saline solution (118 mM NaCl, 4.7 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM KH_2PO_4, 11 mM glucose and 20 mM HEPES, pH 7.4) before fluorescence was monitored on a PTI M-series spectrofluorometer with dual excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. $[Ca^{2+}]_i$ was calculated from the fluorescence ratio as described by Grynkiewicz et al. (1985).

2.7. Statistical analysis

Each experiment was performed in duplicate, and data represent the means ± S.E.M. from at least three independent experiments. $P < 0.05$ was considered significant by evaluation of the data with Student's *t*-test and/or Dunnett's test. The error bar is omitted when it is within the symbol representing the mean value.

3. Results

3.1. Effects of UTP, 2MeSATP and PMA on [^{3}H]arachidonic acid release

To study the mechanism of arachidonic acid release in response to P_2 receptor agonists, bovine pulmonary artery endothelial cells were treated with 2MeSATP and UTP, which act on $P2Y_1$ and $P2Y_2$ receptors, respectively (Chen et al., 1996), for different periods of time and then the radioactivity of [^{3}H]arachidonic acid released into the medium was measured. Fig. 1A shows that both 2MeSATP and UTP at 100 μM increased arachidonic acid release within 60 min in a time-dependent manner. When measured at 30 min, concentration-dependent effects of UTP and 2MeSATP were observed (Fig. 1B). At 100 μM, the arachidonic acid release caused by UTP and 2MeSATP was 326 ± 21% ($n = 3$) and 239 ± 6% ($n = 3$) of basal release, respectively. Addition of ATP (100 μM), the non-selective P_2 receptor agonist, increased arachidonic acid release to 362 ± 14% ($n = 3$) of basal release (data not shown). Since in this cell type, we have previously shown that 100 μM is the maximal concentration of UTP, 2MeSATP and ATP needed to elicit phosphoinositide turnover and that ATP-induced phosphoinositide breakdown occurs after the activation of both $P2Y_1$ and $P2Y_2$ receptors (Chen et al., 1996), we just tested here the arachidonic acid response to ATP at 100 μM to confirm the similar maximal arachidonic acid responses observed for ATP and UTP. Neither the P2X receptor-selective agonist, α,β-methylene ATP, nor the P_1 receptor agonist, adenosine, caused significant arachidonic acid release (data not shown).

In bovine pulmonary artery endothelial cells, addition of PMA at 10 nM–1 μM also led to the release of [^{3}H]arachidonic acid in a concentration-dependent manner

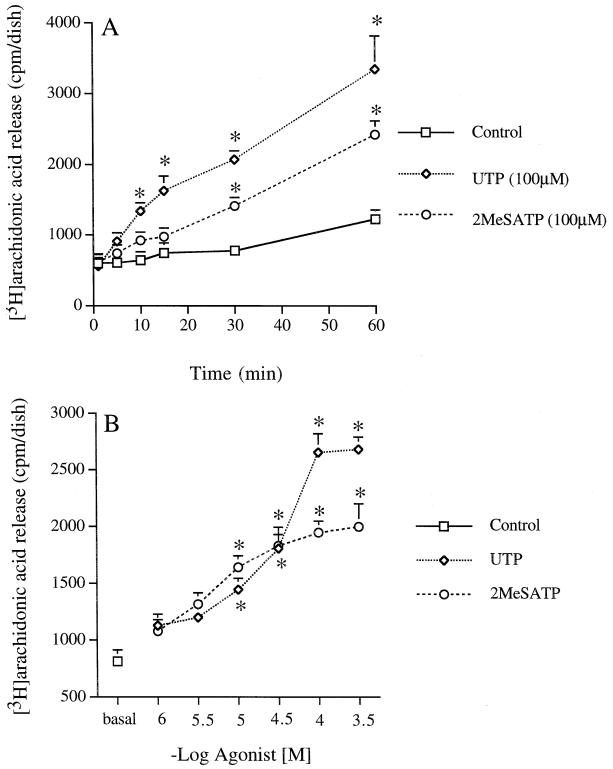

Fig. 1. UTP- and 2MeSATP-induced arachidonic acid release in bovine pulmonary artery endothelial cells. (A) Cells labeled with [^{3}H]arachidonic acid were washed with serum-free MEM containing 0.5% fatty acid-free bovine serum albumin, and then treated with vehicle, 100 μM UTP or 100 μM 2MeSATP for the time indicated. (B) Concentration-dependent effects of UTP and 2MeSATP on [^{3}H]arachidonic acid release within 30 min. After stimulation, the [^{3}H]arachidonic acid release in the medium was measured. The data represent the means ± S.E.M. from three experiments performed in duplicate. * Using Dunnett's test, $P < 0.05$ was considered significant as compared to the control [^{3}H]arachidonic acid release without nucleotide treatment.

(Fig. 2), suggesting the existence of a protein kinase C-dependent regulation of arachidonic acid release in bovine pulmonary artery endothelial cells. The maximal increase was 232 ± 7% ($n = 3$) of basal release, which was achieved by a 30-min treatment with 300–1000 nM PMA.

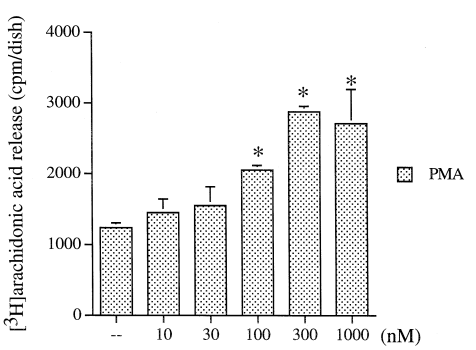

Fig. 2. Stimulatory effects of PMA on [^{3}H]arachidonic acid release. Cells were treated with 10–1000 nM PMA for 30 min and then the [^{3}H]arachidonic acid release in the medium was measured. The data represent the means ± S.E.M. from three experiments performed in duplicate. * Using Dunnett's test, $P < 0.05$ as compared to the response without PMA treatment.

3.2. Ca^{2+}-dependent activation of cytosolic phospholipase A_2

To investigate the role of intracellular Ca^{2+} in arachidonic acid release, cells were incubated in culture medium containing 4 mM EGTA for 5 min, by which $[Ca^{2+}]_i$ of bovine pulmonary artery endothelial cells was reduced from 108 ± 5 to 86 ± 7 nM ($n = 3$). Under this condition, we found that the arachidonic acid release in response to UTP (100 μM), 2MeSATP (100 μM) and PMA (1 μM) was abolished (Fig. 3A). The Ca^{2+}-mediated arachidonic acid release elicited by Ca^{2+}-elevating agents, such as thapsigargin (1 μM, an inhibitor of endoplasmic reticulum Ca^{2+}-pump) and ionomycin (1 μM, a Ca^{2+} ionophore), was also prevented in the absence of extracellular Ca^{2+} (Fig. 3A).

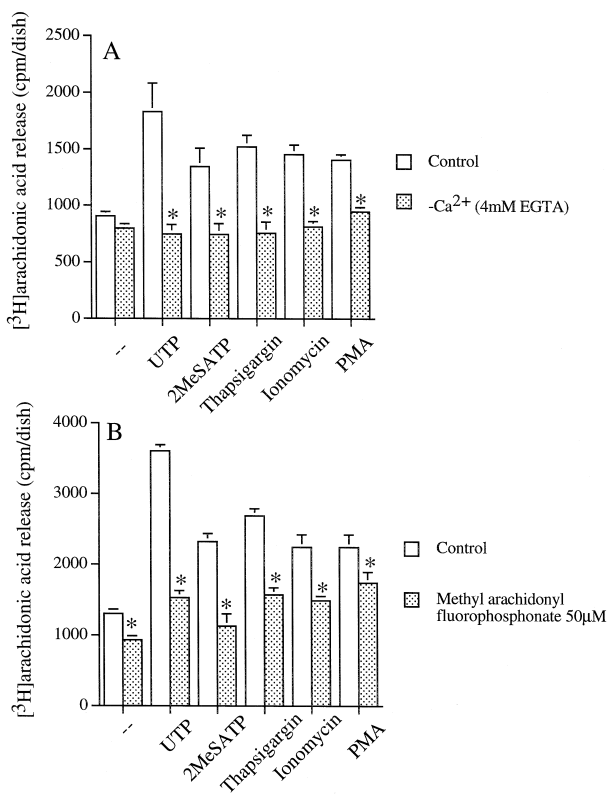

Fig. 3. Ca^{2+}-dependent and methyl arachidonyl fluorophosphonate-sensitive [^{3}H]arachidonic acid release. Cells were incubated in MEM containing 4 mM EGTA for 5 min (A) or pretreated with 50 μM methyl arachidonyl fluorophosphonate for 20 min (B), and then were stimulated with UTP (100 μM), 2MeSATP (100 μM), thapsigargin (1 μM), ionomycin (1 μM) or PMA (1 μM) for another 30 min. The radioactivity of [^{3}H]arachidonic acid in the medium was measured. The data represent the means ± S.E.M. from at least three experiments performed in duplicate. * $P < 0.05$ as compared to the response without EGTA (A) or methyl arachidonyl fluorophosphonate (B) treatment.

To confirm that the stimuli-induced arachidonic acid release resulted from the activation of cytosolic phospholipase A_2, we tested the effects of methyl arachidonyl fluorophosphonate, an inhibitor of cytosolic phospholipase A_2 (Huang et al., 1996). Fig. 3B shows that pretreatment with 50 μM of methyl arachidonyl fluorophosphonate inhibited the basal as well as the five stimuli-induced arachidonic acid release. Thus, these data suggest that cytosolic phospholipase A_2 mediates arachidonic acid release from bovine pulmonary artery endothelial cells.

3.3. Protein kinase C-dependent potentiation of cytosolic phospholipase A_2 activation and negative regulation of Ca^{2+} mobilization

In view of the stimulating effect of PMA on arachidonic acid release (Fig. 2) and the phosphoinositide stimulation by P_2 purinoceptor activation (Chen et al., 1996), we determined the contribution of protein kinase C signalling to P_2 receptor-mediated arachidonic acid release. To do this, the effects of three protein kinase C inhibitors on the responses induced by the P_2 receptor agonists, thapsigargin, ionomycin and PMA, were compared. As shown in Fig. 4, we found that staurosporine (1 μM, a non-selective inhibitor of Ser/Thr protein kinases) and Ro 31-8220 (1 μM, a selective protein kinase C inhibitor) (Beltman et al., 1996) reduced the arachidonic acid responses to UTP, 2MeSATP and PMA, with a greater inhibition being seen for staurosporine than for Ro 31-8220. In contrast, both inhibitors had no effect on the responses to thapsigargin and ionomycin. Go 6976 (a selective inhibitor of conventional protein kinase C α, β and γ) (Martiny-Baron et al., 1993) at 1 μM did not significantly affect the arachidonic acid release elicited by all five stimuli.

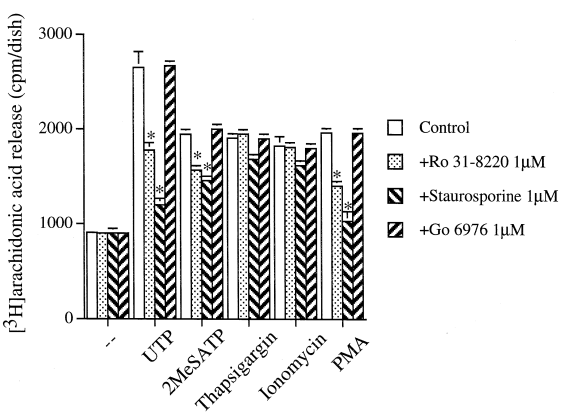

Fig. 4. Effects of protein kinase inhibitors on stimuli-induced [^{3}H]arachidonic acid release. Cells pretreated with vehicle, staurosporine (1 μM), Ro 31-8220 (1 μM) or Go 6976 (1 μM) for 20 min were challenged with UTP (100 μM), 2MeSATP (100 μM), thapsigargin (1 μM), ionomycin (1 μM) or PMA (1 μM). After 30 min the radioactivity of [^{3}H]arachidonic acid in the medium was measured. The data represent the means ± S.E.M. from at least three experiments performed in duplicate. $^*P < 0.05$ as compared to the stimuli response without drug pretreatment.

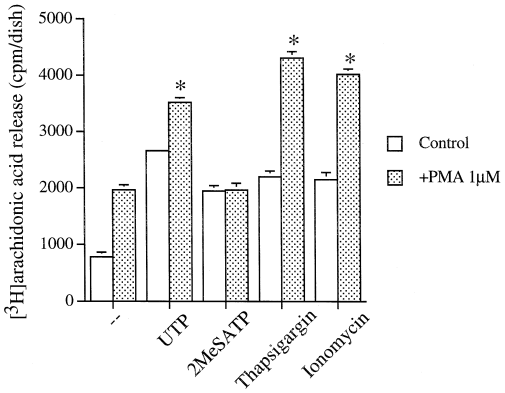

Fig. 5. Effects of protein kinase C activation on stimuli-induced [^{3}H]arachidonic acid release. Cells were pretreated with vehicle or 1 μM PMA for 20 min followed by the addition of 100 μM UTP, 100 μM 2MeSATP, 1 μM thapsigargin or 1 μM ionomycin. The data represent the means ± S.E.M. from at least three experiments performed in duplicate. $^*P < 0.05$ as compared to the stimuli response without PMA pretreatment.

Since activation of protein kinase C by phorbol ester has been shown to cause a synergistic (or priming) effect on agonist-induced arachidonic acid release, we examined the arachidonic acid release from bovine pulmonary artery endothelial cells after combined addition of PMA (1 μM) and various agents. As shown in Fig. 5, PMA indeed caused an additive increase in arachidonic acid release induced by UTP, thapsigargin or ionomycin. Interestingly, however, the arachidonic acid response to 2MeSATP (100 μM) was unaffected by PMA addition.

To investigate the ineffectiveness of PMA on the $P2Y_1$-linked arachidonic acid response, we examined the role of protein kinase C on stimuli-induced Ca^{2+} mobilization, which is essential for cytosolic phospholipase A_2 activation. By using Fura-II/AM to measure $[Ca^{2+}]_i$, we observed biphasic increases in $[Ca^{2+}]_i$ in response to 2MeSATP and UTP, as we previously reported (Chen et al., 1996). Here, we found that the $[Ca^{2+}]_i$ increase elicited by 2MeSATP was prominently attenuated by the presence of 1 μM PMA, while PMA only slightly reduced the response to UTP and did not affect that to thapsigargin. As summarized in Fig. 6, PMA inhibited the peak and sustained phases of the 2MeSATP-induced $[Ca^{2+}]_i$ rise by 57 ± 1% ($n = 4$) and 58 ± 1% ($n = 4$), respectively, inhibited the peak phase of the UTP response by 23 ± 2% ($n = 6$), but had no effect on the sustained phase of the UTP response. These data suggest that in 2MeSATP-treated cells, the stimulatory role of PMA on cytosolic phospholipase A_2 activation, if any, for example through phosphorylation of cytosolic phospholipase A_2, may be cancelled out by the potent attenuation of the $[Ca^{2+}]_i$ rise, which results in the lack of effect of PMA on the $P2Y_1$ receptor-linked arachidonic acid response.

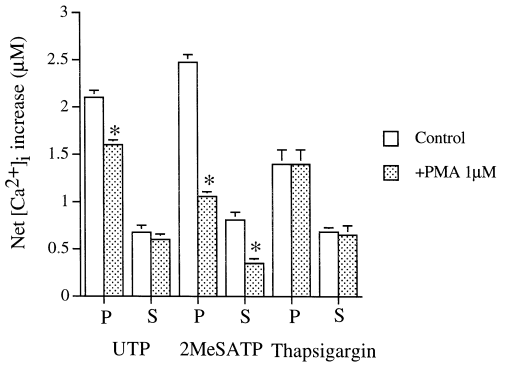

Fig. 6. Effects of PMA on stimuli-induced $[Ca^{2+}]_i$ increase. Cells were treated with 100 μM 2MeSATP, 100 μM UTP or 1 μM thapsigargin in the absence or presence of 1 μM PMA. The effects of PMA pretreatment on the peak (P) and sustained (S) increase (3 min after stimuli addition) in $[Ca^{2+}]_i$ are summarized. The basal level of $[Ca^{2+}]_i$ was 110 ± 7 nM ($n = 9$). The data represent the means ± S.E.M. from three to six experiments. $^*P < 0.05$ as compared to the response without PMA presence.

3.4. Protein kinase C ε involved in the cytosolic phospholipase A_2 activation

To further identify which protein kinase C isoform is involved in cytosolic phospholipase A_2 activation in bovine pulmonary artery endothelial cells, we measured the immunoreactivity of protein kinase C isoforms following PMA stimulation. Using specific antibodies against 12 protein kinase C isoforms, we found that, in bovine pulmonary artery endothelial cells, protein kinase C βI, ε, λ and μ isoforms were expressed (Fig. 7A), while the others (i.e., α, βII, γ, δ, θ, ζ, ι and η) were undetectable (data not shown). When bovine pulmonary artery endothelial cells were treated with 1 μM PMA for up to 24 h, we found that the immunoreactivities of βI and ε were time dependently down-regulated (Fig. 7B), while those of λ and μ were unchanged (data not shown). After 4 h of

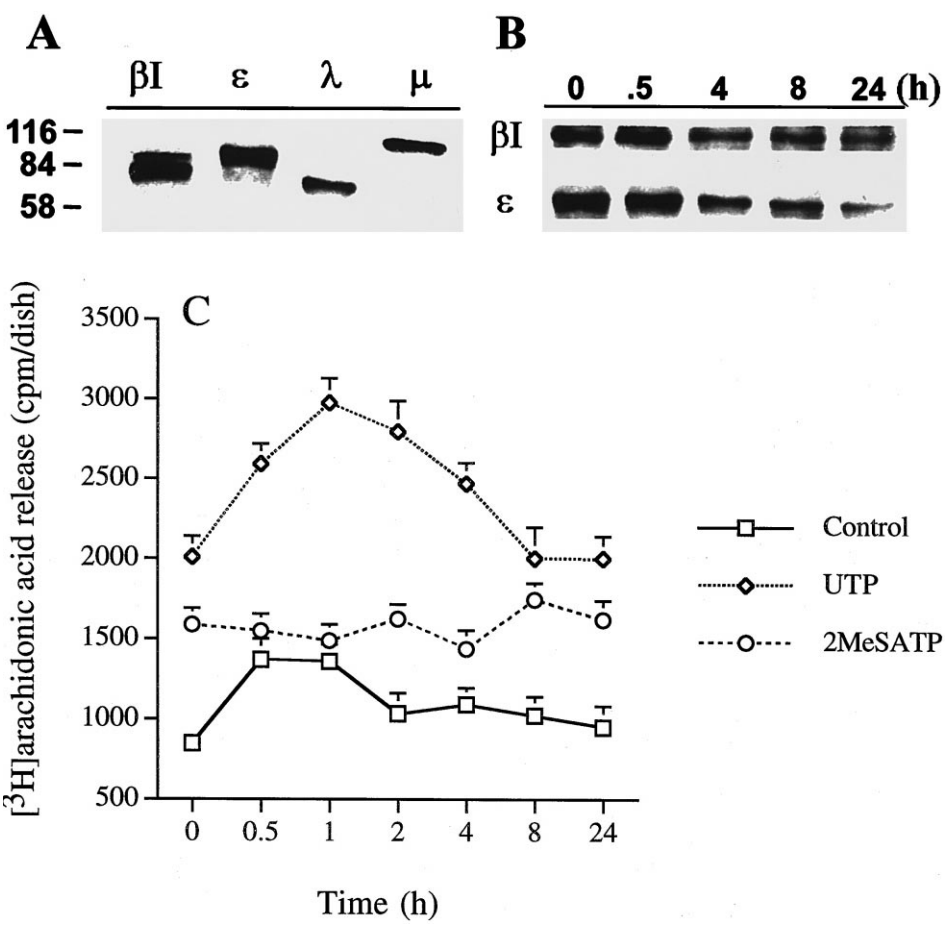

Fig. 7. PMA-induced down-regulation of protein kinase C isoforms and potentiation of stimuli-triggered arachidonic acid release. (A) The expression of protein kinase C isoforms in bovine pulmonary artery endothelial cells was determined by Western blot using protein kinase C isoform-specific antibodies, and four protein kinase C isoforms expressed in bovine pulmonary artery endothelial cells are shown. (B) Cells were treated with 1 μM PMA for 0.5, 4, 8 or 24 h, and then the immunoreactivity of the protein kinase C isoforms was determined. (C) Cells without or with 1 μM PMA pretreatment for different periods were stimulated with vehicle, 100 μM UTP or 100 μM 2MeSATP for 30 min. Then the radioactivity of [^{3}H]arachidonic acid in the medium was measured. The data represent the means ± S.E.M. from three experiments performed in duplicate.

treatment, the levels of βI and ε were reduced by 43 ± 5 and 68 ± 8% ($n = 3$), respectively.

The effect of protein kinase C down-regulation on arachidonic acid release induced by purinergic agents is shown in Fig. 7C. Consistent with the stimulatory effect of PMA in UTP-treated cells, short-term pretreatment (up to 1 h) with PMA increased UTP-induced arachidonic acid release, which was followed by a time-dependent decline to the control level after 8 h of PMA pretreatment (Fig. 5). This decline in response roughly correlated with the down-regulation of protein kinase C βI and ε, suggesting the role of these protein kinase Cs in phospholipase A_2 activation. The arachidonic acid response caused by PMA alone also reached its maximal effect within 1 h. PMA pretreatment had no effect on 2MeSATP-induced arachidonic acid release (Fig. 7C).

3.5. Extracellular signal-regulated protein kinase-dependent cytosolic phospholipase A_2 phosphorylation

To determine the positive regulation of cytosolic phospholipase A_2 activity by extracellular signal-regulated protein kinases, we treated cells with PD 098059 (30 μM), a selective inhibitor of mitogen-activated protein kinase (Dudley et al., 1995), prior to the addition of various stimuli. As shown in Fig. 8A, the arachidonic acid release evoked by UTP and 2MeSATP was inhibited by 31 ± 2 and 23 ± 2% ($n = 4$), respectively, while the response to

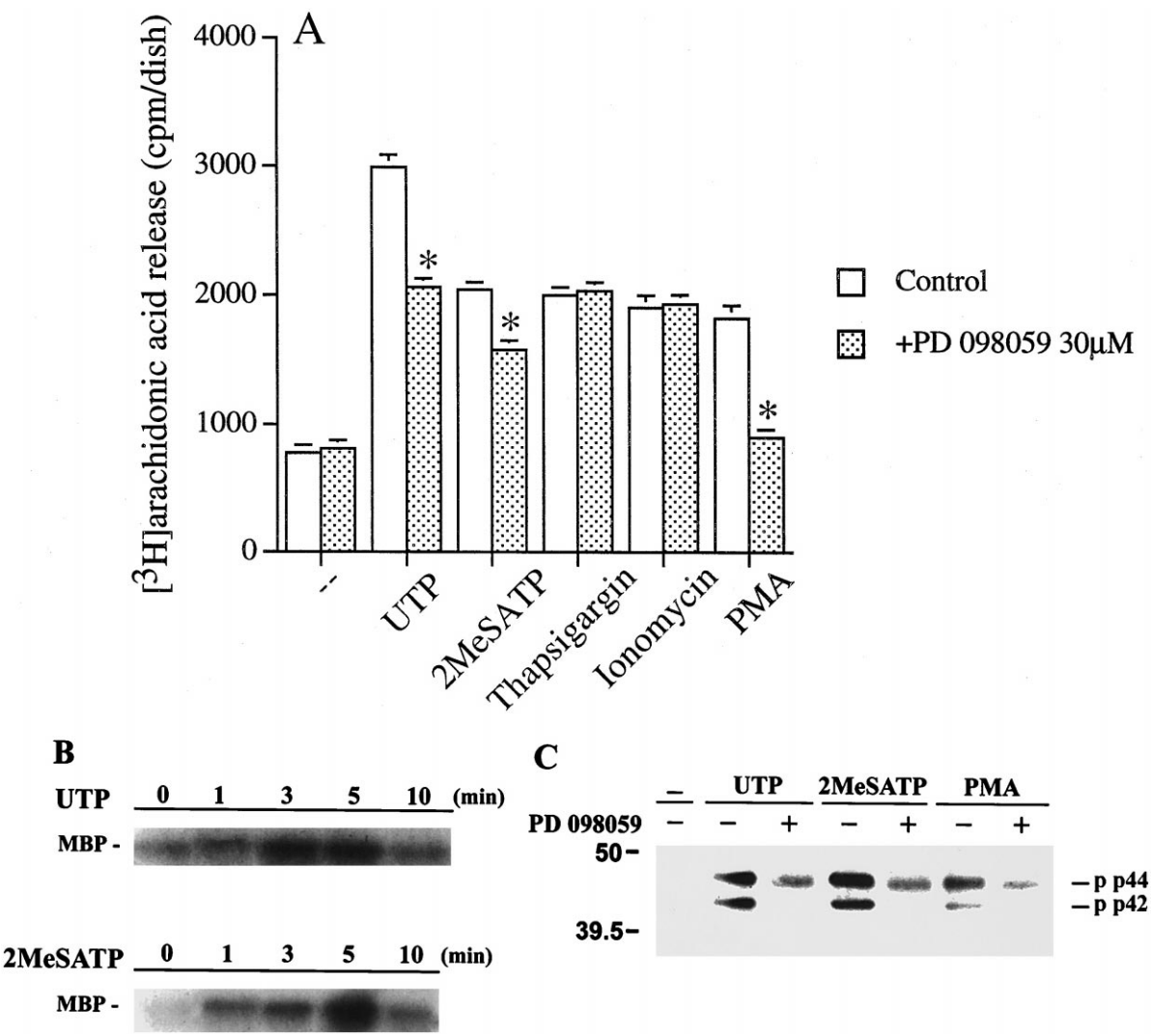

Fig. 8. UTP, 2MeSATP and PMA-induced arachidonic acid release depend on extracellular signal-regulated protein kinase activation. (A) Cells were pretreated with 30 μM PD 098059 for 20 min and then stimulated with UTP (100 μM), 2MeSATP (100 μM), thapsigargin (1 μM), ionomycin (1 μM) or PMA (1 μM) for 30 min. $^*P < 0.05$ as compared to the response without PD 098059 treatment. (B) Extracellular signal-regulated protein kinase activity after UTP (100 μM) or 2MeSATP (100 μM) treatment for different time periods was measured as described in Section 2. (C) Cells pretreated with vehicle or PD 098059 (30 μM) for 20 min were stimulated with UTP, 2MeSATP or PMA for another 5 min. The immunoreactivity of phosphorylated extracellular signal-regulated protein kinases was measured with antibodies specific for their phosphorylated forms. These results are representative of three experiments.

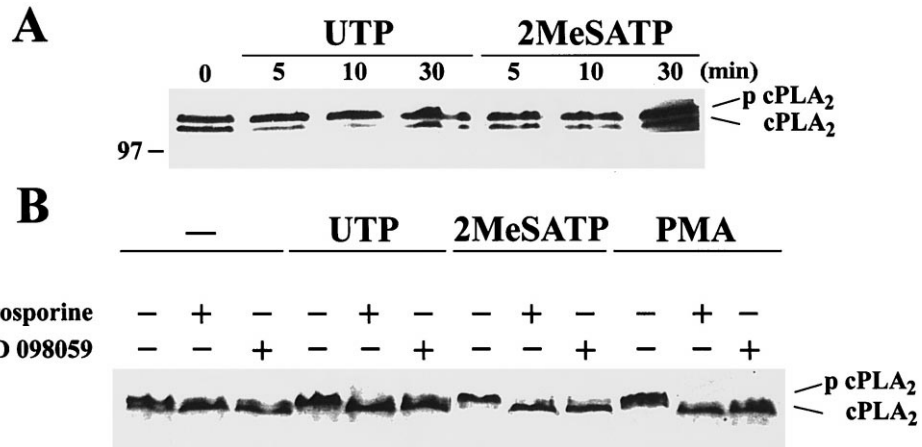

Fig. 9. Stimuli-induced phosphorylation of cytosolic phospholipase A_2. Bovine pulmonary artery endothelial cells at 80–90% confluency were quiescent for 2 days, and then treated with 2MeSATP or UTP, each at 100 μM, or 1 μM PMA. In (B), cells were pretreated with 1 μM staurosporine or 30 μM PD 098059 for 20 min followed by the addition of each stimulus. These traces are representative of three experiments.

PMA was completely abolished. Thapsigargin- and ionomycin-mediated responses remained unchanged. To assess the activity of extracellular signal-regulated protein kinase directly, we performed the immune-complex kinase assay as well as immunoblotting analysis using antibody against phosphorylated extracellular signal-regulated protein kinases. Fig. 8B shows that both 2MeSATP and UTP at 100 μM rapidly increased extracellular signal-regulated protein kinase activity at 1 min; the peak response was reached at 3–5 min. Western blot analysis also showed that extracellular signal-regulated protein kinase 1 and 2 were tyrosine phosphorylated in bovine pulmonary artery endothelial cells after exposure to 2MeSATP, UTP and PMA for 5 min (Fig. 8C). As expected, all of these effects were greatly reduced by PD 098059 (30 μM).

Subsequent experiments were performed to examine the change in the mobility of cytosolic phospholipase A_2 as it has been shown that cytosolic phospholipase A_2 migrates as a doublet on SDS–PAGE, with the phosphorylated protein migrating more slowly, corresponding to an increase in its enzymatic activity (Murayama et al., 1990; Lin et al., 1992). As shown in Fig. 9A, within 5 min, 2MeSATP and UTP changed the migration of cytosolic phospholipase A_2, an effect which was maintained for at least 30 min. Moreover, PMA (1 μM) also caused cytosolic phospholipase A_2 phosphorylation, and its effect, together with that of 2MeSATP and UTP, was inhibited by the presence of 1 μM staurosporine or 30 μM PD 098059 (Fig. 9B). Thapsigargin and ionomycin failed to alter the mobility of cytosolic phospholipase A_2 (data not shown).

4. Discussion

ATP via P_2 purinoceptors has been shown to stimulate prostacyclin release in endothelial cells, but the mechanism underlying the activation has not been clearly defined (Lustig et al., 1992; Paglin et al., 1993; Patel et al., 1996a). In this study, we, for the first time, provide direct evidence that stimulation of both $P2Y_1$ and $P2Y_2$ receptors can activate cytosolic phospholipase A_2. The mechanism of cytosolic phospholipase A_2 activation requires elevation of $[Ca^{2+}]_i$ as well as activation of protein kinase C ε-dependent extracellular signal-regulated protein kinases, leading to the phosphorylation of cytosolic phospholipase A_2.

In this study, several lines of evidence led us to believe that cytosolic phospholipase A_2 is responsible for the 2MeSATP- and UTP-stimulated arachidonic acid release. First, a cytosolic phospholipase A_2 inhibitor, methyl arachidonyl fluorophosphonate (Huang et al., 1996), blocked 2MeSATP- and UTP-stimulated arachidonic acid release. In contrast, an inhibitor of diacylglycerol lipase, RHC 80267 (Kozawa et al., 1997), had no significant effect (data not shown), arguing against the possibility that the arachidonic acid released from stimulated bovine pulmonary artery endothelial cells is derived from phosphoinositide-dependent diacylglycerol generation. Second, Ca^{2+} appeared to be essential and sufficient for arachidonic acid release from bovine pulmonary artery endothelial cells, since EGTA could block the response and the Ca^{2+}-elevating agents, thapsigargin and ionomycin, stimulated arachidonic acid release (Fig. 3). These data are consistent with the involvement of calcium-dependent cytosolic phospholipase A_2. It is interesting to note that the observation that an increase in intracellular calcium was sufficient for cytosolic phospholipase A_2 activation in bovine pulmonary artery endothelial cells is also valid for RAW 264.7 and peritoneal macrophages (Qiu et al., 1993; Lin and Chen, 1998), but not for BAC1.2F5 macrophages (Xu et al., 1994).

With regard to the involvement of protein kinase C in cytosolic phospholipase A_2 activation, our results indicate that both 2MeSATP- and UTP-induced arachidonic acid release were partially inhibited by the non-selective in-

hibitors of protein kinase C, staurosporine and Ro 31-8220. Thus, we suggest that protein kinase C activation is an important component of cytosolic phospholipase A_2 stimulation by phospholipase C-linked receptors. The stimulation of cytosolic phospholipase A_2 by thapsigargin and ionomycin was unchanged by protein kinase C inhibitors, suggesting the existence of another Ca^{2+}-associated, but protein kinase C-unrelated, signalling pathway in the stimulation of cytosolic phospholipase A_2. This pathway is likely mediated through the calcium-dependent translocation of cytosolic phospholipase A_2 from the cytosol to the membrane (Clark et al., 1991; Schievella et al., 1995). Thus, we conclude that both inositol 1,4,5-trisphosphate-elicited Ca^{2+} mobilization and diacylglycerol-linked protein kinase C activation, which are the two branching signals of phosphoinositide turnover, play a coordinated role in cytosolic phospholipase A_2 activation.

Despite the presence of four protein kinase C isoforms, βI, ε, λ and μ, in bovine pulmonary artery endothelial cells, we suggest that protein kinase C ε is involved in cytosolic phospholipase A_2 activation. This is primarily due to the lack of effect of Go 6976, a selective inhibitor of Ca^{2+}- and diacylglycerol-sensitive protein kinase C isoforms (i.e., α, βI, βII and γ) (Martiny-Baron et al., 1993), on stimuli-induced arachidonic acid release. This finding is distinct from our previous observation that protein kinase C β has a crucial role in the mediation of cytosolic phospholipase A_2 activation by UTP in murine RAW 264.7 macrophages (Lin and Chen, 1998). Thus, we suggest that the permissive role of particular protein kinase C isoforms in cytosolic phospholipase A_2 regulation is cell type-specific. Consistent with this, it has been reported that protein kinase C α and β participate in phorbol ester-mediated arachidonic acid release by MDCK and liver macrophages, respectively (Goodman et al., 1991; Duyster et al., 1993).

It has been reported previously that cytosolic phospholipase A_2 becomes phosphorylated in different cell types upon stimulation with endotoxin, cytokines, phorbol ester and neurotransmitters. In bovine pulmonary artery endothelial cells, we observed the phosphorylation of cytosolic phospholipase A_2 after incubation with $P2Y_1$ and $P2Y_2$ receptor agonists and PMA, as evidenced by a change in the mobility of cytosolic phospholipase A_2 on SDS/PAGE. The shift in cytosolic phospholipase A_2 became detectable 5 min after addition of these agents. However, there was no apparent change in the mobility of cytosolic phospholipase A_2 after treatment with thapsigargin and ionomycin. This is in contrast to our previous results obtained with Chinese hamster ovary cells over-expressing cytosolic phospholipase A_2 (Lin et al., 1992) and mouse peritoneal macrophages (Qiu et al., 1993), where the Ca^{2+} ionophore was shown to stimulate the phosphorylation of cytosolic phospholipase A_2. Furthermore, our data indicate the requirement of extracellular signal-regulated protein kinases for full activation of cytosolic phospholipase A_2. The complete inhibition of the PMA-induced arachidonic acid response by PD 098059 ruled out the participation of the extracellular signal-regulated protein kinase-independent protein kinase C pathway in endothelial cells. Supporting this notion, protein kinase C in vitro can directly phosphorylate cytosolic phospholipase A_2 (Nemenoff et al., 1993) and the results of our previous study with RAW 264.7 macrophages also indicates this possibility (Lin and Chen, 1998). Moreover, the inability of thapsigargin and ionomycin to phosphorylate cytosolic phospholipase A_2, and the lack of effect of PD 098059 on their responses, further support the existence of an extracellular signal-regulated protein kinase-independent, but Ca^{2+}-dependent process for cytosolic phospholipase A_2 activation.

In this study, we found that $P2Y_1$ receptor-mediated Ca^{2+} mobilization is negatively regulated by PMA, and to a greater extent than the $P2Y_2$ receptor-induced response (Fig. 6). These results confirmed our previous findings that the phosphoinositide response, as assessed by the accumulation of inositol monophosphate, elicited by 2MeSATP is substantially attenuated by PMA (with 80% inhibition), but that elicited by UTP is reduced by 50% (Chen et al., 1996). This illustrates the differential control of the two purinoceptor responses by protein kinase C.

In conclusion, we demonstrate for the first time that both $P2Y_1$ and $P2Y_2$ purinoceptors in bovine pulmonary artery endothelial cells can cause phosphorylation and activation of cytosolic phospholipase A_2. This action is due to the increase in intracellular Ca^{2+} mobilization and activation of extracellular signal-regulated protein kinase via a protein kinase C ε-dependent pathway, with the combination of both mechanisms leading to the full activation of cytosolic phospholipase A_2. The released arachidonic acid as well as its metabolites can thus act as first and second messengers to mediate the physiological functions of P_2 purinoceptors in the endothelium.

Acknowledgements

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