Biochemical Pharmacology, Vol. 43, No. 6, pp. 1235–1242, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

STIMULATION OF PHOSPHATIDIC ACID SYNTHESIS IN BOVINE AORTIC ENDOTHELIAL CELLS IN RESPONSE TO ACTIVATION OF P_2-PURINERGIC RECEPTORS

JOHN PURKISS, P. JANE OWEN, J. ALISON JONES and MICHAEL R. BOARDER*
Department of Pharmacology and Therapeutics, University of Leicester, P.O. Box 138, Medical Sciences Building, University Road, Leicester LE1 9HN, U.K.

(*Received* 22 *October* 1991; *accepted* 9 *December* 1991)

Abstract—In this study we used the bovine thoracic aorta endothelial cell line AG 4762 and primary bovine aortic endothelial cells to investigate the formation of phosphatidic acid (PA) in response to activation of P_2-purinergic receptors. 2-Methylthio ATP (2MeSATP) stimulated the formation of [^{32}P]-PA in bovine aortic endothelial cells labelled with $^{32}P_i$ for 2.5 hr. A comparison of the response to other ATP analogues suggests that this was mediated via a P_{2Y}-purinergic receptor. Using various agonists at 30 μM there was a correlation between the formation of [^{32}P]PA and of total inositol phosphates in the presence of lithium. The 2MeSATP-stimulated accumulation of [^{32}P]PA showed an initial high rate, followed by a more sustained slower rate. The initial response was independent of extracellular calcium while the later response was dependent on calcium influx. The protein kinase C stimulator phorbol myristate acetate (PMA) produced only a very small enhancement of [^{32}P]PA accumulation compared to 2MeSATP. The 2MeSATP stimulation of both inositol phosphates and [^{32}P]PA was almost eliminated by the presence of PMA. Using cells prelabelled with [^{3}H]methylcholine 2MeSATP produced only a small non-significant enhancement of [^{3}H]choline formation; PMA by contrast formed a much larger amount of [^{3}H]choline. There was no evidence of a change in [^{3}H]phosphocholine. The dissociation between phospholipase D (PLD) activation and [^{32}P]PA accumulation and the correlation between stimulation of [^{32}P]PA accumulation and phospholipase C (PLC) activation all suggest that, using this protocol for labelling cells, the principle route of the stimulation of formation of [^{32}P]PA is via the activation of PLC followed by metabolism of diacylglycerol (DAG) by DAG kinase. These results show that activation of P_{2Y}-purinergic receptors on aortic endothelial cells leads to the formation of phosphatidic acid and that both PLD and PLC pathways are likely to contribute to this response.

It is now well documented that vascular endothelial cells play an active role in the regulation of local functions such as vascular permeability, production of vascular smooth muscle relaxing factors and contracting factors, blood clotting cascades, and platelet adhesion and activation. The influence which endothelial cells exert is under the control of a variety of cell surface receptors including those which respond to ATP and ADP, the P_2-purinergic receptors [1]. Several studies have shown that these receptors are linked to the production of two important biochemical indices of endothelial cell function: endothelin-derived relaxing factor [2–5] and prostacyclin [6, 7]. On the basis of relative agonist potencies two principle subtypes of P_2 receptors have been proposed, P_{2X} and P_{2Y} [8], and it has been shown in studies on aortic endothelial cells that these responses are mediated by the P_{2Y} subtype [4, 5, 7].

In searching for the receptor effector mechanism(s) involved it was discovered that activation of phospholipase C (PLC†) and generation of inositol phosphate and DAG with raised free intracellular calcium were consquences of P_{2Y} receptor activation [9]. It has been suggested that the elevation in free intracellular calcium and the subsequent activation of phospholipase A_2 is responsible for the P_2 receptor augmentation of prostacyclin release [10, 11], and also that protein kinase C activation by DAG mediates both prostacyclin and endothelium-derived relaxing factor release [12]. We have confirmed recently that aortic endothelial cells produce inositol phosphates in response to different analogues of ATP with a potency consistent with the presence of a P_{2Y} receptor subtype, but provide evidence that P_2 receptors on cells of different vascular origin may be neither P_{2X} or P_{2Y} [13].

In addition to PLC, two reports have provided evidence indicating that activation of P_{2Y} receptors on aortic and pulmonary artery endothelial cells may be linked to PLD [14, 15]. Action of this enzyme leads directly to production of PA as the lipid fragment and not DAG as is the case with PLC. Both enzymes can however lead to the increased synthesis of PA, since DAG produced by PLC action may be converted to PA by DAG kinase. Furthermore, PLD may be activated secondarily as a consequence of PLC production of DAG (as shown for example in granulocytes [16]), again leading to PA production. PA is of interest in part as an index of phospholipase activities and a metabolite of DAG,

* Corresponding author. Tel. (0533) 522930; FAX (0533) 523013.

† Abbreviations: PLC, phospholipase C; DAG, diacylglycerol; PLD, phospholipase D; PA, phosphatidic acid; 2MeSATP, 2-methylthioATP; ATPγS, adenosine 5′-*O*-(3-thiotrisphosphate); PMA, phorbol myristate acetate; BAE, bovine aortic endothelial cell primary culture; BSS, balanced salt solution.

but also because second messenger potential of its own has been reported (e.g. Refs 17–19).

In this paper we show that stimulation of P_2-purinergic receptors of aortic endothelial cells leads to increased formation of phosphatidic acid. We characterise this response and provide evidence that the stimulation of P_{2Y}-receptor subtypes involves both activation of PLC and PLD, both of which will contribute to the receptor-mediated enhancement of intracellular PA.

MATERIALS AND METHODS

Cell cultures. Two types of cell culture, both of bovine thoracic aorta endothelial origin, were used in these studies. Firstly, the cell line AG4762, a non-clonal cell line obtained from the National Institute of Aging (Bethesda, MD, U.S.A.), was used as described in a previous study [13]. The medium was Dulbecco's modified Eagle's with 10% fetal calf serum, 27 mg/mL glutamine and 1/100 mL 100 × gentamycin, fungizone, penicillin and streptomycin; cells were used between passages 17 and 19. Secondly, cultures of endothelial cells were prepared from fresh bovine thoracic aorta essentially as described in Ref. 20. These are referred to as BAE cells. Briefly, one intact and undamaged aorta was clamped at one end, arteries were ligated, and the inside was washed gently with 200 mL BSS (NaCl, 125 mM; KCL, 5.4 mM; $NaHCO_3$, 16.2 mM; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid, 30 mM; NaH_2PO_4, 1 mM; $MgSO_4$, 0.8 mM; $CaCl_2$, 1.8 mM; glucose, 5.5 mM; buffered to pH 7.4 with NaOH and gassed with 95% O_2/5% CO_2). The aorta, held vertical, was then almost filled with BSS with 0.5 mg/mL collagenase (Sigma Chemical Co., Poole, U.K., type I) and incubated for 20 min at 37°, after which it was washed twice gently with BSS and half filled with complete cell culture medium (D-valine minimal essential medium, 5% fetal calf serum, 15% newborn calf serum, with glutamine, gentamycin, fungizone, penicillin and streptomycin as above). The aorta, closed at both ends and held horizontally was shaken vigorously several times. The resultant suspension of small sheets of endothelial cells was broken up into single or small clumps of cells by trituration with an 18 g needle and seeded directly into 24-well multiwell plates. After one day the medium was replaced with Dulbecco's modified Eagle's medium with 5% fetal calf serum and 5% newborn calf serum. The cells were confluent after 4–6 days in culture, forming a homogeneous cell population with the classical cobblestone features of cultured endothelial cells. The wells were free of any discernable non-endothelial contamination and shown to be factor VIII positive by immunohistochemistry. In some cases this primary culture was passaged once prior to use of cells.

Measurement of [^{32}P]*PA*. Cells washed in phosphate free BSS were incubated with $^{32}P_i$ at 0.25 MBq/mL, for 2.5 hr in most experiments. Phosphate (1 mM) was returned to the medium 1 min before cells were stimulated by removal of the label medium and replacement with the incubation medium. For experiments where extracellular calcium was buffered, BSS was made up without added calcium and buffered, using 5 μM Quin 2 and up to 10 μM EGTA, to 50–100 nM free CA^{2+} as measured by fluorescence at 490 nm (excitation at 340 nM). Stimulation was stopped by removal of supernatant and extraction of cells into 0.9 mL of ice-cold methanol: 1 M HCl (5:4) with 0.5 mL $CHCl_3$. After drying down under N_2, separation with a 1-steroyl, 2-arachidonyl-PA standard was on oxalate-coated silica gel 60 TLC plates and development was in 9:2:5 ethylacetate: acetic acid: 2,2,4-trimethylpentane. PA was visualized by I_2 staining and spots were scraped into 1 mL $CHCL_3$ and counted by liquid scintillation counting. We showed that there was little quenching caused by $CHCl_3$ under our conditions of counting. With this procedure the radioactivity formed a single peak associated with the PA spot, essentially free of radioactivity on either side. Confirmation that the radioactivity was in PA was undertaken in a separate series of experiments using adrenal chromaffin cells, by sequential TLC and deacylation, followed by HPLC, as described elsewhere [21].

In some experiments in which the incorporation of label into ATP was monitored, the aqueous phase was retained and an aliquot injected onto a 25 cm Partisil 10 SAX column (Technicol, Stockport, Cheshire, U.K.) and eluted with a gradient of ammonium phosphate. About half the radioactivity applied to the column eluted with an ATP standard.

Use of [^{3}H]*glycerol-labelled cells*. Multiwell plates with confluent cells were loaded with 0.37 MBq/mL [^{3}H]glycerol for 48 hr in medium M199. Cells were washed twice with BSS. After further incubation for 2 hr, the loaded cells were stimulated as described. The reaction was stopped by the removal of supernatant and extraction of cells with 0.25 mL of cold methanol. After scraping, the wells were washed in 0.25 mL methanol/0.45 mL H_2O. Lipids were separated into 0.5 mL $CHCl_3$, dried under N_2 and separated on oxalate-coated silica gel TLC plates developed first in ethylacetate: acetic acid: trimethylpentane (9:2:5) and second in hexane: diethyl ether: methanol: acetic acid (90:20:3:2). Internal standards of PA and DAG were identified by iodine staining and the spots scraped and scintillation counted.

Use of [^{3}H]*choline-labelled cells*. Cells were loaded according to Pirotton *et al.* [14]. Confluent cells were incubated for 24 hr in multiwells with 0.5 mL/well of M199 containing 0.074 MBq/mL [*methyl*-^{3}H]-choline chloride. After washing with BSS cells were stimulated as required in 0.5 mL BSS for 20 min. The reaction was stopped with 0.5 mL cold methanol, wells were washed with 0.5 mL methanol/0.4 mL H_2O and 1 mL $CHCl_3$ was added. The aqueous phase was separated [22] on small cation exchange columns (1 mL of Dowex-50W) into fractions containing glycerophosphocholine, phosphocholine and choline. Aliquots of these fractions were scintillation counted.

Use of [^{3}H]*inositol-labelled cells*. To measure incorporation of label into total inositol phosphates, multiwell plates with confluent cells were incubated at 37° for 40–48 hr with 0.037 MBq of *myo*-[2-^{3}H]-inositol in 0.5 mL of loading medium (M199, 27 mg/

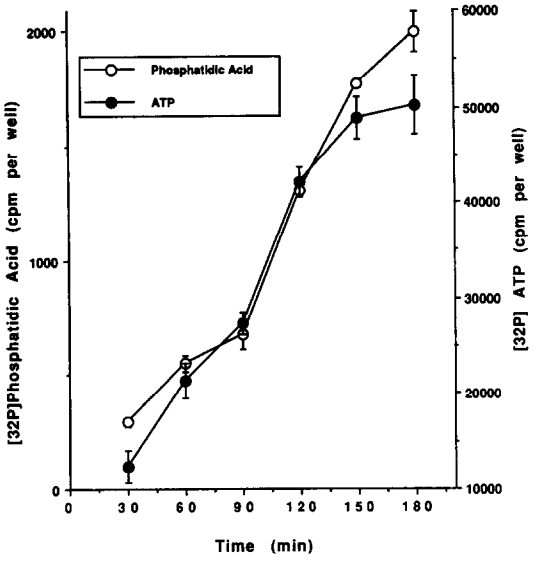

Fig. 1. Accumulation of ^{32}P in PA and in ATP in extracts from unstimulated AG4762 cells labelled with ^{32}P for the time shown. Data points are means ± SEM (N = 4) from a single experiment representative of three.

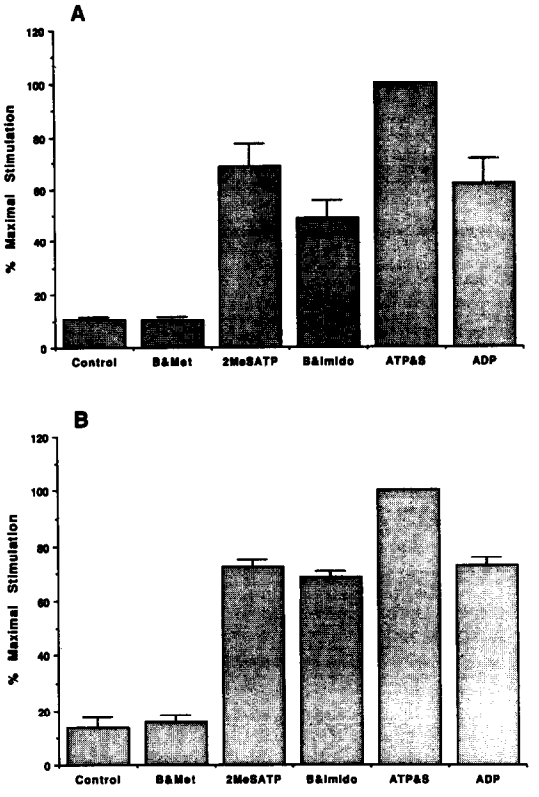

Fig. 2. Accumulation of [^{32}P]PA and [^{3}H]inositol phosphates in stimulated AG 4762 cells. Stimulation was with 30 μM of β-γ-methylene ATP (B & Met), 2MeSATP, β,γ-imido ATP (B & imido), ATPγS (ATP & S) and ADP. The stimulations for the [^{32}P]PA measurements were 5 min, those for the [^{3}H]inositol phosphate measurements were 30 min. Results are means ± SEM of three separate experiments each in quadruplicate; the data have been expressed as a percentage of the maximum (ATPγS) stimulation. ATPγS gave a 993 ± 77.5% over basal stimulation for [^{32}P]PA and a 862 ± 128% over basal stimulation for [^{3}H]inositol phosphates (N = 12 in each case). The stimulations by 2MeSATP, 2 β-γ-imido ATP, ATPγS and ADP were all significant at $P < 0.05$.

100 mL glutamine and fungizone, penicillin and streptomycin). Cells were then incubated in BSS with 10 mM LiCl for 10 min prior to stimulation for 30 min at 37° in the presence of lithium. The reaction was stopped by the addition of 1 mL ice-cold methanol and after lipid extraction, water soluble inositol phosphates were extracted batchwise onto Dowex-1 (Cl^- form) and eluted with 1 M HCl for liquid scintillation counting.

To measure incorporation into individual inositol phosphates, cells in 3.5 mM dishes were incubated in loading medium with 0.22 MBq [^{3}H]inositol at 37° for 40–48 hr. Cells were then incubated for the time indicated in BSS (without lithium) at 37°; preincubation with phorbol ester where indicated was for the prior 10 min, with the phorbol ester also present during the incubation period. The cells were extracted into 1 M trichloroacetic acid, the acid was removed by ether extraction and the inositol phosphates separated on small AG1 anion exchange columns as described previously [13].

Materials. Cell culture medium, additives and platics were from Gibco (Paisley, U.K.). *myo*-[2-^{3}H]Inositol (15 Ci/mmol) was from New England Nuclear (Stevenage, U.K.), other radiochemicals were from Amersham International (Amersham, U.K.). 2MeSATP was from Research Biochemicals (Semat Ltd, St. Albans, U.K.). Other chemicals were from the Sigma Chemical Co. (Poole, U.K.) or Fisons plc. (Loughborough, U.K.). TLC plates were from Whatman (Maidstone, U.K.).

RESULTS

Formation of [^{32}P]*PA*

The studies involving labelling of cells with [^{32}P]orthophosphate, followed by stimulation and extraction of [^{32}P]PA, were undertaken mainly with the AG4762 bovine thoracic aorta endothelial cell line. An example of preliminary studies investigating the rate of incorporation of label into phospholipids and ATP is shown in Fig. 1. Cells were labelled, as described in Materials and Methods but for varying periods of time. Extraction into methanol: HCl: chloroform followed with no stimulation. The aqueous phase as analysed for label in ATP by HPLC, and the organic phase was fractionated by TLC to yield the label in PA. These results show that label incorporation into ATP and PA continued with a similar time-course through 3 hr. Other experiments have shown that incorporation of label into ATP continues beyond 3 hr and that the incorporation of label into the total lipid phase, and into phosphatidylcholine, follows the same time course as into PA (data not shown). Subsequent studies used 2.5-hr labelling except where indicated.

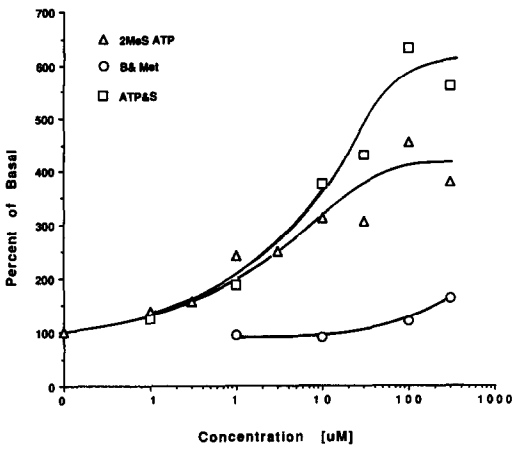

Fig. 3. Concentration–response relationship for 5-min stimulation of AG 4762 cells with 30 μM of 2MeSATP, β,γ-methylene ATP (B & Met) or ATPγS (ATP & S). Results are means of four separate experiments each in quadruplicate or triplicate, expressed as a percent of maximal stimulation (ATPγS, 100 μM).

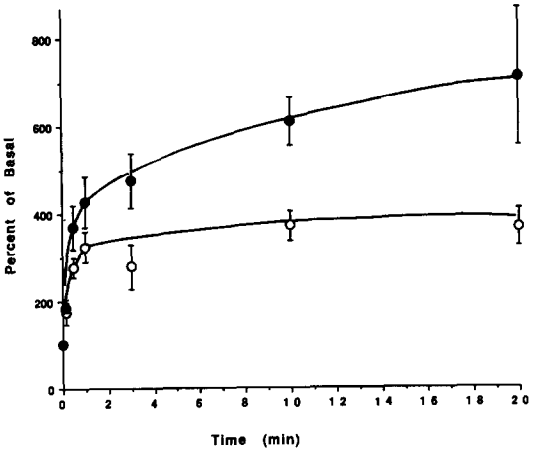

Fig. 4. Time-course of 2MeSATP (30 μM) stimulation of accumulation of [^{32}P]PA in AG 4762 cells, in presence of 1.8 mM Ca^{2+} (closed circles) or 100 nM Ca^{2+} (open circles). Mean ± SEM of four separate experiments each with triplicate determinations.

Figure 2A shows that 2MeSATP (a P_{2Y}-specific agonist) stimulated the production of labelled PA in $^{32}P_i$-labelled AG 4762 cells. A similar magnitude of response was seen with β,γ-imido-ATP and ADP, while ATPγS gave a larger response, up to eight to nine times the basal level of [^{32}P]PA. However, the P_{2X}-specific agonist β,γ-methylene-ATP failed to elicit an enhanced accumulation of label in [^{32}P]PA. Figure 2B enables a direct comparison with the stimulation of label into total inositol phosphates in the presence of lithium, which may be taken as an index of phosphoinositide-specific PLC activity. The relative agonist response was the same for PLC activation as for [^{32}P]PA production. The dose–response relationships for the production of [^{32}P]PA with respect to 2MeSATP, ATPγS and β,γ-methylene-ATP are shown in Fig. 3. The maximal response for 2MeSATP was less than that for ATPγS, and again β-γ-methylene-ATP failed to elicit a response.

Using 2MeSATP as agonist, the time-course of [^{32}P]PA formation is shown in Fig. 4. In the presence of 1.8 mM extracellular Ca^{2+} the response was biphasic, with a rapid phase in the first minute followed by a slower sustained rise continuing through 20 min. When extracellular Ca^{2+} was buffered to 50–100 nM, this slow sustained response was lost leaving a similar rapid phase which reached a plateau at 1 min, which was characteristically 3–4-fold higher than the control (unstimulated) level.

In order to investigate further a role for calcium influx we treated cells with the calcium ionophore A23187. In the same series of experiments we investigated the effect of PMA, a protein kinase C-stimulating phorbol ester, and looked at the consequences of combining A23187 with PMA, since they may have a synergistic effect. From the data shown in Fig. 5A using AG 4762 cells it can be seen that PMA and A23187 separately produced only a small stimulation of accumulation of [^{32}P]PA compared to 30 μM 2MeSATP. Furthermore, when PMA and A23187 were combined the effect was additive and not synergistic, and was still a small stimulation over control (65%) compared to that of 2MeSATP (251%). A similar series of results with BAE cells are shown in Fig. 5B. Again PMA produced only a very small response compared to 2MeSATP, while BAE cells were different from AG 4762 cemls in that the A23187 response was much larger.

In several reports, short (e.g. 10 min) pretreatment with PMA followed by stimulation with PMA has been shown to attenuate agonist-enhanced formation of inositol phosphates. We pursued studies to see if PMA would reduce the effect of 2MeSATP on the accumulation of inositol phosphates and to see what the corresponding effect on [^{32}P]PA accumulation would be. Fig. 6A shows that PMA treatment eliminated effectively the enhanced accumulation of $InsP_1$, $InsP_2$, $InsP_3$ and $InsP_4$ in response to 2MeSATP. Fig. 6B shows that PMA has a similar affect on the 2MeSATP stimulation of [^{32}P]PA accumulation, leading to a massive reduction in the response.

Formation of [^{3}H]*choline*

Cells were labelled for 24 hr with [^{3}H]choline to label the phosphatidycholine, and the effect of subsequent stimulation on [^{3}H]choline, [^{3}H]-phosphocholine and [^{3}H]glycerophosphocholine was studied. Throughout these experiments there was no effect on [^{3}H]phosphocholine or [^{3}H]glycero-phosphocholine. Figure 7A shows that PMA and A23187 stimulated the formation of [^{3}H]choline in AG4762 cells. Stimulation of AG4762 cells with 2MeSATP produced an enhancement of [^{3}H]choline production which was smaller than that caused by PMA. No response to β,γ-methylene-ATP was seen. With BAE cells (Fig. 7B) only A23187 produced a

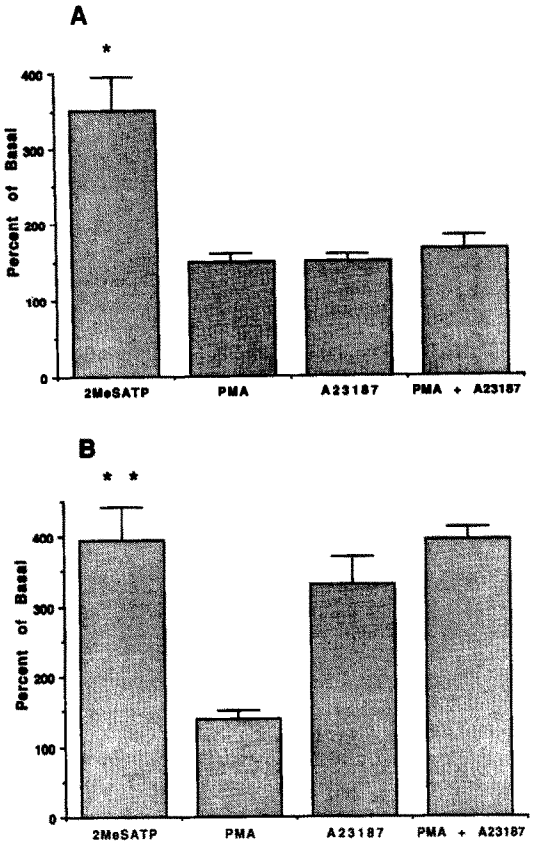

Fig. 5. Stimulation of accumulation of [^{32}P]PA in cell stimulated (5 min) with 10 μM 2MeSATP, 100 nM PMA, 5 μM A23187 or a combination of PMA and A23187 expressed as a percentage of unstimulated controls. (A) AG 4762 cells. Data are means ± SEM of four separate experiments each of which was in quadruplicate. *Stimulation by 2MeSATP significantly different from PMA plus A23187, $P < 0.01$ (paired t-test). (B) BAE cells. Data are means ± SEM of three separate experiments each of which was in quadruplicate. **Stimulation by 2MeSATP significantly different from PMA alone ($P < 0.01$) but not from A23187 alone or with PMA (paired t-test).

significant stimulation. The mean enhancement with PMA was not significant, while 2MeSATP produced no response.

Formation of [^{3}H]*DAG and* [^{3}H]*PA in cells labelled with* [^{3}H]*glycerol*

Cells were labelled with [^{3}H]glycerol and stimulated for varying periods of time with 2MeSATP, and the labelled DAG and PA were extracted, separated on TLC and counted. Figure 8 shows that the [^{3}H]DAG elevation in response to the agonist was biphasic with the initial rapid rise to about double the control level being complete in about 1 min followed by a second peak at about 3 min resulting in a rise sustained for the duration of the experiment (10 min). [^{3}H]PA showed a similar pattern of response: there was no clear separation in the time-course of the responses of the two metabolites.

DISCUSSION

The data presented in this paper establish PA as a major product of P_{2Y} activation in vascular endothelial cells. The results are consistent with two further conclusions. Firstly, that under the conditions of our experiment the majority of [^{32}P]PA formed by P_{2Y} receptor agonist activation is by the sequential action of PLC and DAG kinase. Secondly that PLD is also activated by the action of the P_{2Y} receptor.

In the absence of any differential antagonists the distinction between P_{2Y} and P_{2X} receptors has been by relative agonist potency. Here we have not attempted to produce a full range of dose–response curves characterizing the [^{32}P]PA response: we have undertaken this previously for AG 4762 cells with respect to [^{3}H]inositol phosphate accumulation [13]. However, the effectiveness of 2MeSATP, but not β-γ-methylene ATP, is inconsistent with a P_{2X} receptor, but along with the response to ATPγS and ADP is consistent with a P_{2Y} receptor. The time-courses of response in the presence and absence of extracellular Ca^{2+} show a rapid phase which is largely independent of extracellular calcium and a slow and sustained calcium-dependent phase. Previous studies have shown that P_{2Y} receptor activation can stimulate calcium influx. It seems likely therefore that the rapid calcium-independent phase represents direct activation of phospholipase(s) while the slow sustained phase is a consequence of calcium influx, a suggestion encouraged by the observation that the calcium ionophore A23187 can stimulate [^{32}P]PA accumulation.

The experiments with PMA are illuminating with respect to the relative involvement of PLD and PLC. It has been suggested that PLD can be maximally activated by PMA [16]. The observation that PMA can induce only a very small increase in counts in [^{32}P]PA compared to 2MeSATP would indicate therefore that the latter must be mainly by a non-PLD pathway. Comparing the effects of PMA (using AG 4762 cells) on [^{32}P]PA and [^{3}H]choline accumulation provides support for this position since PMA produces a larger response than 2MeSATP for [^{3}H]choline accumulation while the reverse is true for the [^{32}P]PA response. This indicates that the [^{32}P]PA accumulation in response to 2MeSATP cannot come principally from PLD action on phosphatidylcholine. We assume that the stimulated [^{3}H]choline production occurs directly by action of PLD and not phosphatase action on phosphocholine produced by PLC since no stimulations of [^{3}H]-phosphocholine were seen at any time investigated.

The other experimental procedure using PMA reported here is the inhibition of the 2MeSATP stimulation of PLC, seen as a reduction in the formation of inositol phosphates almost to the basal level. If the stimulation of [^{32}P]PA accumulation was sustained under these conditions then it would indicate a clear dissociation from the phosphoinositide-directed PLC activity. However, the majority of the [^{32}P]PA response is also lost. This seems incompatible with this part of the

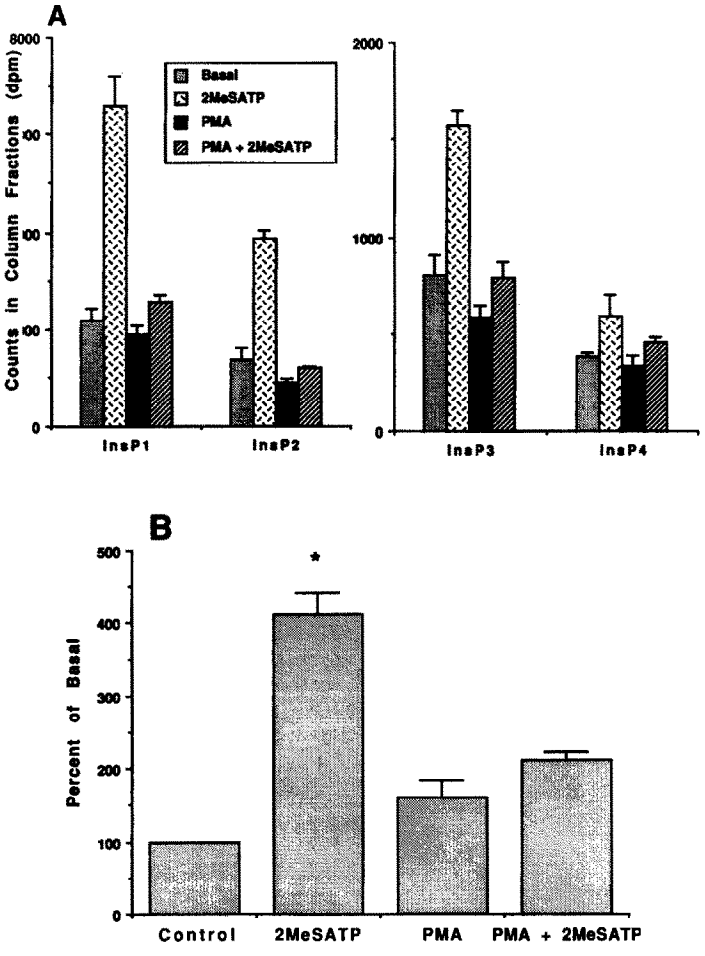

Fig. 6. Effect of 10 min preincubation with PMA. (A) Effect of a short (10 min) preincubation with PMA (100 nM) on accumulation of [^{3}H]inositol phosphates stimulated for 2 min by 2MeSATP (10 μM). The results shown for each inositol phosphate are from unstimulated (basal) incubations, 2MeSATP-stimulated incubations, preincubation and incubation with PMA alone and preincubation with PMA and incubation with PMA plus 2MeSATP; mean ± SEM of three determinations from a single experiment representative of three. (B) As for A except accumulation of [^{32}P]PA was measured for each condition and the results expressed as % increase over unstimulated control counts are means ± SEM from four separate experiments each undertaken as triplicate or quadruplicate determinations. *Significantly different from PMA plus 2MeSATP $P < 0.01$ (t-test).

response being by PLD, since this enzyme is stimulated, not inhibited, by the phorbol ester [16]. The most likely explanation for these observations is that the majority of the 2MeSATP-stimulated [^{32}P]PA produced is inhibited by PMA because it originated from the PLC hydrolysis of inositol phospholipid.

Considering the effects of PMA, it is interesting to speculate that a consequence of PLC activation generating DAG leading to inhibition of PLC may be to enhance the relative contribution of PLD. The use of [^{3}H]choline-labelled cells to investigate the agonist-induced breakdown of choline phospholipids showed a stimulation of AG 4762 cells, but not BAE cells in response to 2MeSATP. The cells are different in that the AG 4762 are a highly passaged pure endothelial cell line, while the BAE cells are either not passaged or are passaged only once. How this relates to a difference in response is not clear; the failure to see a response in BAE cells was unexpected in view of two earlier reports [14, 15]. However, in both these studies the agonists reported did not include 2MeSATP, which was chosen in the present study because it is the most P_{2Y} selective.

A limitation of the $^{32}P_i$ labelling procedure used here is that the relative specific activity of the γ-phosphate on ATP and of the relevant pool and species of phospholipid is not known. However, studies additional to those reported here using cells labelled for 24 hr, such that the various putative precursors are presumably closer to equilibrium labelling, also show PA production by both PLC and PLD pathways. A considerable advantage of the $^{32}P_i$-labelling procedure is that phospholipids are labelled regardless of their headgroup, and that precursors of both the PLC- and the PLD-mediated

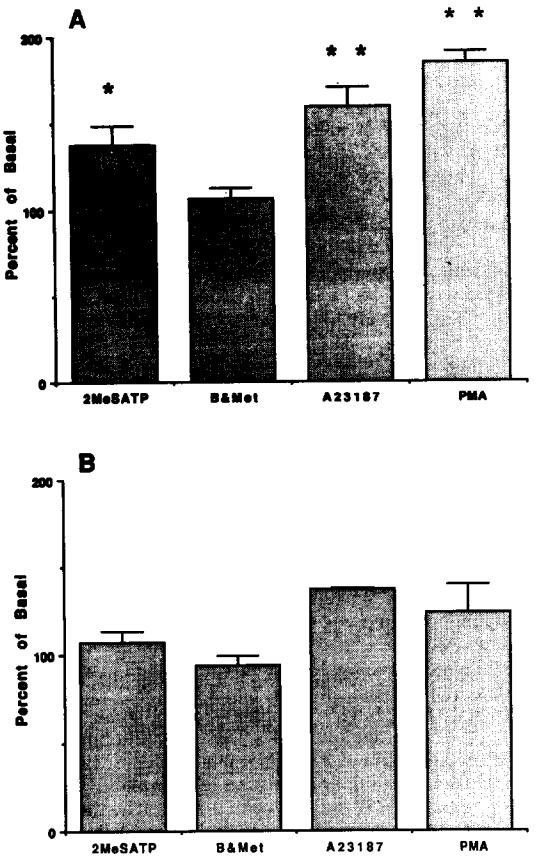

Fig. 7. Accumulation of [^{3}H]choline. (A) AG 4762 and (B) BAE cells were stimulated for 20 min with 30 μM of 2MeSATP, 30 μM β,γ-methylene ATP (B & Met), 5 μM A23187 or 100 nM PMA. Results are % increase over unstimulated controls from four separate experiments each carried out with quadruplicate determinations. *Stimulation with 2MeSATP was significantly smaller than that with PMA ($P < 0.01$, t-test); **Significantly different from control ($P < 0.01$).

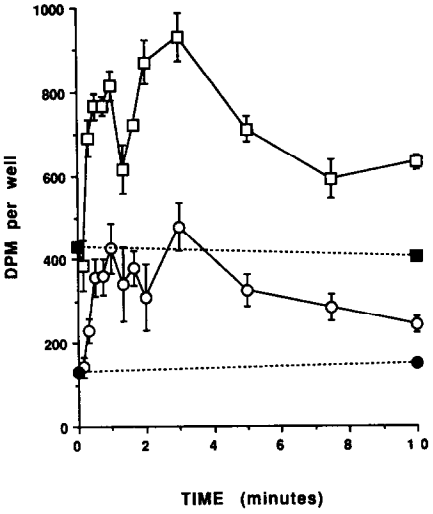

Fig. 8. Accumulation of [^{3}H]DAG and [^{3}H]PA in glycerol loaded cells. Stimulation with 30 μM 2MeSATP (open symbols) was for the times indicated: [^{3}H]DAG (squares), [^{3}H]PA (circles). The closed symbols are the counts from unstimulated cells. The results are means ± SEM of quadruplicate determinations of a single experiment representative of three.

pathways are labelled, enabling their assessment following activation with agonists.

In summary, the results reported here show: (1) activation of bovine aortic endothelial P_{2Y}-purinergic (and not P_{2X}) receptors leads to the enhanced accumulation of [^{32}P]PA; (2) the initial phase of this stimulation is independent of extracellular Ca^{2+}, but a lower level and more sustained stimulation is dependent on calcium influx; (3) PMA and A23187 have a greater effect than 2MeSATP on PLD hydrolysis of phosphatidylcholine, while the reverse is true for labelled PA accumulation; (4) both P_{2Y}-stimulated PA production and inositol lipid breakdown can be inhibited by PMA. These results lead to the conclusion that effects of ATP and ADP on BAE cells are mediated by both PLC and PLD activation following P_{2Y}-receptor occupation by an agonist, but that using the procedures described here the principle effect is via the PLC pathway. Further studies are underway using different times of labelling with $^{32}P_i$, and distinguishing between PLC and PLD by stimulating the cells in the presence of butanol, as described for neuronal cells in a recent publication [23].

Acknowledgements—We thank The Wellcome Trust for financial support and Lyn McCarthy for manuscript preparation.

REFERENCES

1. Boeynaems J-M and Pearson JD, P_2-purinoceptors on vascular endothelial cells: physiological significance and transduction mechanisms. *Trends Pharmacol Sci* **11**: 34–37, 1990.
2. De Mey JG and Vanhoutte PM, Role of the endothelin in cholinergic and purinergic relaxation of isolated canine femoral arteries. *J Physiol* **316**: 347–355, 1981.
3. Gordon JL and Martin W, Endothelium dependent relaxation of the pig aorta: relationship to stimulation of Rb efflux from isolated endothelial cells. *Br J Pharmacol* **79**: 531–541, 1983.
4. Houston DA, Burnstock G and Vanhoutte PM, Different P_2-purinergic receptor subtypes of endothelium and smooth muscle in canine blood vessels. *J Pharmacol Exp Ther* **241**: 501–509, 1987.
5. Martin W, Cusack NJ, Carleton JS and Gordon JL, Specificity of P_2-purinoceptor that mediates endothelium dependent relaxation of the pig aorta. *Eur J Pharmacol* **108**: 295–299, 1984.
6. Pearson TD, Slakey LL and Gordon JL, Stimulation of prostaglandin production through purinoceptors on cultured porcine endothelial cells. *Biochem J* **214**: 273–276, 1987.
7. Needham L, Cusack NJ, Pearson JD and Gordon JL, Characteristics of the P_2-purinoceptor that mediates prosacyclin production by pig aorta endothelial cells. *Eur J Pharmacol* **134**: 199–209, 1987.
8. Burnstock G and Kennedy C, Is there a basis for

distinguishing two types of P_2 receptors. *Gen Pharmacol* **16**: 433–440, 1985.
9. Pirotton S, Raspe E, Demolle D, Erneux C and Boeynaems J-M, Involvement of inositol 1,4,5-trisphosphate and calcium in the action of adenine nucleotides on aortic endothelial cells. *J Biol Chem* **262**: 17461–17466, 1987.
10. Boeynaems J-M, Pirotton S, Van Coevorden A, Rapse E, Demolle D and Erneaux C, P_2-purinergic receptors in vascular endothelial cells: from concept to reality. *J Recept Res* **8**: 121–132, 1988.
11. Carter TD, Hallam TJ, Cusack NJ and Pearson JD, Regulation of P_2-purinoceptor-mediated prostacyclin release from human endothelial cells by cytoplasmic calcium concentration. *Br J Pharmacol* **95**: 1181–1190, 1988.
12. De Nucci G, Gryglewski RJ, Warner TD and Vane JR, Receptor mediated release of endothelium derived relaxing factor and prostacyclin from bovine aortic endothelial cells is coupled. *Proc Natl Acad Sci USA* **85**: 2334–2338, 1988.
13. Allsup DJ and Boarder MR, Comparison of P_2-purinergic responses of aortic endothelial cells with those of adrenal medulla: evidence for heterogeneity of receptor subtype and of inositol phosphate response. *Mol Pharmacol* **38**: 84–91, 1990.
14. Pirotton S, Rabaye B, Lagneau C and Boeynaems J-M, Adenine nucleotides modulate phosphatidylcholine metabolism in aortic endothelial cells. *J Cell Physiol* **142**: 449–457, 1990.
15. Martin TW and Michaelis K, P_2-purinergic agonists stimulate phosphodiesteratic cleavage of phosphatidylcholine in endothelial cells: evidence for activation of phospholipase D. *J Biol Chem* **264**: 8847–8856, 1989.
16. Billah MM, Pai JK, Mullman TJ, Egan RW and Siegel MI, Regulation of phospholipase D in HL-60 granulocytes: activation by phorbol esters, diacylglyceride and calcium ionophor via protein kinase-independent mechanisms. *J Biol Chem* **264**: 9069–9076, 1989.
17. Moolenaar WH, Kruijer W, Tilly BC, Verloan I, Bierman AJ and de Laal SW, Growth factor-like action of phosphatidic acid. *Nature (Lond)* **323**: 171–173, 1986.
18. Murayama T and Ui M, Phosphatidic acid may stimulate membrane receptors mediating adenylate cyclase inhibition and phospholipase breakdown in 3T3 fibroblasts. *J Biol Chem* **262**: 5522–5529, 1987.
19. Van Corven EJ, Goenink A, Jalink K, Eichholz T and Moolenaar WH, Lysophosphatidate-induced cell proliferation: identification and dissection of signalling pathways mediated by G-proteins. *Cell* **59**: 45–54, 1989.
20. Booyse FM, Sedlak BJ and Rafelson ME, Culture of arterial endothelial cells: characterisation and growth of bovine aortic cells. *Thromb Diath Haemorrh* **34**: 825–839, 1975.
21. Owen PJ and Boarder MR, Influence of bradykinin on diacylglycerol and phosphatidic acid accumulation in cultured bovine adrenal chromaffin cells. *J Neurochem* **57**: 760–768, 1991.
22. Cook SJ and Wakelam MJO, Analysis of the water soluble products of phosphatidylcholine breakdown by ion exchange chromatography: bombesin and TPA stimulate choline generation in Swiss 3T3 cells by a common mechanism. *Biochem J* **263**: 581–587, 1989.
23. Purkiss JR, Murrin RA, Owen PJ and Boarder MR, Lack of phospholipase D activity in chromaffin cells: bradykinin stimulated phosphatidic acid formation involves phospholipase C in chromaffin cells but phospholipase D in PC12 cells. *J Neurochem* **57**: 1084–1087, 1991.