Stimulation of phosphatidate synthesis in endothelial cells in response to P_2 -receptor activation

Evidence for phospholipase C and phospholipase D involvement, phosphatidate and diacylglycerol interconversion and the role of protein kinase C

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To investigate the stimulation of phosphatidic acid formation in bovine aortic endothelial cells by P_2 -purinergic agonists, we labelled AG4762 cells with [32 P]P_i and stimulated in the presence of butanol. Under these conditions phospholipase D generated [32 P]phosphatidylbutanol, whereas the [32 P]phosphatidic acid from phospholipase C and diacylglycerol kinase was unchanged. The action of various purinergic agonists on both [32 P]phosphatidic acid and [32 P]phosphatidylbutanol was consistent with the presence of a P_{2Y} receptor. The stimulation of phospholipase D was dependent on extracellular Ca^{2+} and was mostly transient (completed within 3 min), whereas the initial stimulation of phospholipase C was independent of extracellular Ca^{2+} , followed by a Ca^{2+} -dependent phase. The agonist stimulation of phospholipase D was dependent on protein kinase C, as judged by its sensitivity to the relatively selective protein kinase C inhibitor Ro 31-8220. These results show that purinergic-receptor-mediated stimulation of phosphatidic acid has three phases: an initial Ca^{2+} -independent stimulation of phospholipase C, an early but transient Ca^{2+} - and protein kinase C-dependent stimulation of phospholipase D, and a sustained Ca^{2+} -dependent stimulation of phospholipase C. Using propranolol to inhibit phosphatidate phosphohydrolase, we provide evidence that phosphatidic acid derived from purinergic-receptor-mediated stimulation of the phospholipase C/diacylglycerol kinase route can itself be converted back into diacylglycerol.

INTRODUCTION

The endothelium plays a key regulatory role in several vascular processes, including permeability, smooth-muscle relaxation and contraction, platelet adhesion and activation, and blood-clotting cascades. The participation of endothelial cells in these processes 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]. Two principal subtypes of P_2 -receptors have been proposed on the basis of relative agonist potencies, P_{2X} and P_{2Y} [2]. Aortic endothelial cells are known to possess the P_{2Y} subtype [3,4]. P_{2Y} receptors of aortic endothelium are coupled to a phospholipase C (PLC), activation of which generates inositol trisphosphate and diacylglycerol (DAG) [5]. Inositol trisphosphate formation is temporally associated with an increase in free intracellular Ca^{2+} , derived from both extracellular and intracellular sites [5,6]. The role of raised free intracellular Ca^{2+} in prostacyclin production is clearly documented [7,8], and also the fact that protein kinase C (PKC) activation modulates this process [9,10]. PKC also plays a role in release of endothelium-derived relaxing factor [11]. More recently however, attention has focused on the purinergic-receptor-mediated stimulation of phospholipase D (PLD) in endothelial cells and the production of choline metabolites and phosphatidic acid (PA) as a result of the action of this enzyme on phosphatidylcholine [12–14]. PA can be readily converted into DAG by the action of PA phosphohydrolase, an enzyme active in endothelial cells [15]. Several potential second-messenger functions for PA itself have also been proposed (e.g. [16]).

We have previously reported large accumulations of [32 P]PA after purinergic-receptor-mediated stimulation of [32 P]P_i-labelled endothelial cells [17]. With this procedure, there are two pathways by which agonist stimulation may give rise to [32 P]PA: sequential action of PLC and DAG kinase, or directly by the PLD route mentioned above. In the present paper we show that P_2 -receptor stimulation of aortic endothelial cells leads to the accumulation of PA by activation of both pathways. We characterize the agonist stimulation of these pathways and provide evidence suggesting that PA derived from both routes is readily metabolized to DAG.

EXPERIMENTAL

Materials

H₃[32 P]PO₄ was purchased from ICN-Flow Laboratories (Paisley, Scotland, U.K.). Adenosine 5'-[γ -thio]triphosphate (ATP[S]), adenosine 5'-[β -methylene]triphosphate (p[CH₂]-ppA), ATP and UTP were purchased from Sigma (Poole, Dorset, U.K.). 2-Methylthio-ATP (2MeSATP) was from Research Biochemicals Inc. (Natick, MA, U.S.A.). Ro 31-8220 was given by Dr. Geoffrey Lawton (Roche Products, Welwyn Garden City, Herts., U.K.). Silica-gel G t.l.c. plates (LK 6D) were provided by Whatman (Maidstone, Kent, U.K.). 1-Stearoyl-2-arachidonyl-*sn*-glycerol DAG and PA standards were supplied by Sigma. 1-Stearoyl-2-[1- 14 C]arachidonyl-PBut was given by Dr. R. Bonser (Burroughs Wellcome, Beckenham, Kent, U.K.). Cell-culture supplies were from Gibco (Paisley, Scotland, U.K.).

Abbreviations used: PLC, phospholipase C; DAG, diacylglycerol; PKC, protein kinase C; PLD, phospholipase D; PA, phosphatidic acid; PBut, phosphatidylbutanol; BSS, balanced salt solution; PMA, phorbol 12-myristate 13-acetate; ATP[S], adenosine 5'-[γ -thio]triphosphate; p[CH₂]ppA, adenosine 5'-[β -methylene]triphosphate; 2MeSATP, 2-methylthio-ATP.

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Other chemicals were from Sigma or Fisons (Loughborough, Leics., U.K.).

Cell culture, radiolabelling and treatment

The non-clonal bovine aorta endothelial cell line AG 4762, obtained from the National Institute of Ageing (Bethesda, MD, U.S.A.) was used throughout this study. Cells were maintained in 80 cm³ flasks and passaged by treatment with 0.25 % trypsin. The medium was Dulbecco's modified Eagle's medium with 10 % (v/v) foetal-calf serum, glutamine (27 mg/ml), gentamycin (50 µg/ml), fungizone (2.5 µg/ml), penicillin (100 IU/ml) and streptomycin (100 IU/ml). All experiments were performed with confluent monolayers in 24-well multidishes at passage 18–26.

Experiments were carried out at 37 °C in a balanced salt solution (BSS): NaCl, 125 mM; KCl, 5.4 mM; NaHCO₃, 16.2 mM; Hepes, 30 mM; MgSO₄, 0.8 mM; NaH₂PO₄, 1 mM; CaCl₂, 1.8 mM; glucose, 5.5 mM; buffered to pH 7.4 with NaOH and gassed with O₂/CO₂ (19:1). Cells were labelled by incubation for 24 h with BSS with NaH₂PO₄ omitted and containing H₃[³²P]PO₄ (0.25 MBq/ml; 0.4 ml/well). Optimal conditions of labelling were established in preliminary experiments in which cells labelled for different periods of time were extracted and the lipids separated by t.l.c. into phosphatidylcholine, phosphoinositides plus phosphatidylserine and phosphatidylethanolamine. Each phospholipid was labelled maximally at 24 h with a loading time course which extended to 72 h. Metabolic stability after such incubations was assessed in these and other cell types by incubating under the same conditions but with [³H]inositol in place of [³²P]P_i; robust agonist-stimulated formations of inositol (poly)phosphates were achieved by incubations of 48 h. Morphologically, the cells incubated under these conditions were stable over 48 h.

For stimulation of these labelled cells, the labelling medium was replaced with 0.5 ml of BSS containing agonist with other compounds as required. Incubations were terminated by removal of stimulating solution and immediate addition of 0.5 ml of ice-cold methanol. Cells were scrape-harvested and transferred to Microfuge tubes; the wells were washed with 0.4 ml of water and washings transferred. Lipids were extracted into 0.5 ml of chloroform. After mixing and separation of phases by centrifugation, the aqueous phase was removed. Chloroform extracts were dried down under N₂, redissolved in 80 µl of chloroform/methanol (19:1, v/v) and applied with non-radioactive standards to the pre-absorbent zone of oxalate-treated silica-gel G t.l.c. plates. [¹⁴C]PBut standard was applied to lanes adjacent to samples. Plates were developed in ethyl acetate/acetic acid/2,2,4-trimethylpentane (9:2:5, by vol.). [³²P]PA, [³²P]PBut and [¹⁴C]PBut standard were detected by autoradiography of the plates on Kodak X-Omat film for 48 h. PA standard was detected with I₂ vapour and found to correspond exactly to a spot on the autoradiographs. [³²P]PA and [³²P]PBut spots were found to be well resolved and essentially free of intervening radioactivity. They were scraped into 0.5 ml of chloroform/methanol (19:1, v/v) and counted for radioactivity by liquid-scintillation counting, the chloroform having been found to cause minimal quench of ³²P. The identity of the ³²P co-migrating with PA standard was confirmed in parallel experiments in which the radioactivity from the t.l.c. plate was eluted, deacylated and the glycerophosphate derivative separated by h.p.l.c., as described in [18].

When butanol was present, it was added to a concentration of 50 mM for the last 10 min of the labelling period and was present at 50 mM in the incubation medium. When PKC inhibition was investigated, Ro 31-8220 was added for the last 20 min of labelling and was present in the incubation medium. The final

concentration of dimethyl sulphoxide never exceeded 0.1 %. Propranolol was used to inhibit PA phosphohydrolase at a concentration of 250 µM, and was added to the labelling medium 5 min before stimulation and was present in the incubation medium. In experiments where extracellular Ca²⁺ was buffered, BSS was prepared without added Ca²⁺ and buffered to 50–100 nM free Ca²⁺ with 5 µM-Quin-2 and up to 10 µM-EGTA. Fluorescence was measured at 490 nm with excitation at 340 nm. ³²P-pre-labelled cells were washed twice with BSS without added Ca²⁺ before a 10 min preincubation with 50 mM-butanol prepared in buffered Ca²⁺/BSS. Stimulatory solutions were prepared in buffered Ca²⁺/BSS with 50 mM-butanol.

RESULTS

In preliminary experiments we showed that, when AG 4762 bovine aorta endothelial cells were loaded with [³²P]P_i for 24 h, incubation with 30 µM-ATP[S] led to a several-fold stimulation of [³²P]PA accumulation [17]. In order to distinguish PA derived by sequential action of PLC and DAG kinase from that derived by PLD activation, we have stimulated the [³²P]P_i-loaded cells in the presence of butanol. This takes advantage of the transphosphatidyl transfer reaction unique to PLD [19], in which an alcohol acts as an acceptor of the phosphatidate moiety, leading to the production of a phosphatidylalcohol. Preliminary work established that 50 mM-butanol, a 10-fold more potent acceptor than ethanol [20], gave maximal PBut production under both resting and stimulated conditions. For example, when ³²P accumulating in PBut is expressed as a percentage of radioactivity in PA plus PBut, under stimulated (30 µM-ATP[S]) conditions 3 mM-butanol gave 15.2 %, 10 mM gave 36.9 %, 30 mM gave 46.2 % and 50 mM gave 44.9 % in the form of PBut (means of three determinations).

The studies reported here show that when 50 mM-butanol is present the purinergic agonists cause a stimulation of both [³²P]PA and [³²P]PBut accumulation (Table 1). PLD was therefore active in aortic endothelial cells. The relative ability of the agonists at 30 µM to stimulate [³²P]PBut production followed the same pattern as for [³²P]PA (Table 1), with p[CH₂]₂ppA inactive and ATP[S] the most active. Active phorbol esters such as phorbol 12-myristate 13-acetate (PMA) are widely described as activators of PLD [14]. Here we have compared stimulation of [³²P]PA and [³²P]PBut formation, in the presence of 50 mM-butanol, by 100 nM-PMA and 30 µM-ATP[S] (Table 2). The large [³²P]PBut response to 3 min treatment with 100 nM-PMA is in accordance with previous reports of stimulation of PLD by PMA [12,13]. The differential [³²P]PBut stimulation over [³²P]PA contrasts with the purinergic-agonist stimulation of these two products. A small (less than 2-fold) [³²P]PA stimulation was observed with 100 nM-PMA.

Table 1. Stimulation of [³²P]PA and [³²P]PBut by various agonists

Each compound was at 30 µM with a 3 min stimulation. Data are c.p.m. from a single experiment representative of three separate experiments, as means ± s.e.m., *n* = 4. Significance of difference from unstimulated control: **P* < 0.05, ***P* < 0.001 (Student's *t* test).

Agonist	[³² P]PBut	[³² P]PA
Control	4185 ± 745	6583 ± 433
p[CH ₂] ₂ ppA	4279 ± 348	6617 ± 221
2MeSATP	8562 ± 396*	14985 ± 736**
ATP[S]	11 795 ± 317**	28071 ± 1397**
UTP	8406 ± 472*	19694 ± 639**
ATP	9550 ± 212**	22379 ± 436**

Table 2. Stimulation of [³²P]PA and [³²P]PBut by ATP[S] and PMA

Incubations were for 3 min in the presence of either PMA or ATP[S]. Data were collected from three separate experiments each carried out in triplicate and expressed as fold increase over unstimulated controls (means ± S.E.M., *n* = 3).

	Stimulation (fold over basal)	
	[³² P]PBut	[³² P]PA
PMA (100 nM)	5.72 ± 0.98	1.67 ± 0.21
ATP[S] (30 μM)	2.50 ± 0.17	4.19 ± 0.55

Fig. 1. Effect of increasing concentrations of ATP[S] (3 min incubation) on accumulation of [³²P]PBut (●) and [³²P]PA (○) in the presence of 50 mM-butanol

The data are means ± S.E.M. pooled from three separate experiments, each in triplicate.

ATP[S] at 30 μM consistently produced the largest [³²P]PA response, with or without butanol, and the largest [³²P]PBut response, with butanol, compared with the other purinergic agonists. For this reason it was chosen as the stimulant for the rest of the study. Fig. 1 shows the dose-response relationship for both [³²P]PA and [³²P]PBut to different concentrations of ATP[S]. The EC₅₀ for ATP[S] stimulation of [³²P]PA in the three experiments represented in Fig. 2 was 4.8 ± 0.45 μM, and 2.4 ± 1.9 μM for [³²P]PBut; the two values were not significantly different.

Time-course experiments were undertaken to see if a temporal relationship existed between the activation of PLC ([³²P]PA production) and PLD ([³²P]PBut production). The example shown in Fig. 2 is representative of the three experiments performed. Stimulation of [³²P]PBut accumulation was detectable at the earliest time point (2.51 ± 0.16 times basal). The principal phase of accumulation was finished by 3 min, with an overall stimulation of 3.21 ± 0.17 times basal at 20 min across the three experiments. The initial stimulation of [³²P]PA was reproducible across the three experiments (1.81 ± 0.08 times basal), as was the continued elevation above basal at 20 min (3.89 ± 0.74 times).

Dependence of PLC/DAG kinase and PLD routes on extracellular Ca²⁺ was examined in three parallel experiments illustrated by that shown in Fig. 3. Stimulation by 30 μM-ATP[S] in the presence of 50–100 nM-Ca²⁺ resulted in almost total loss of the PLD response. This is demonstrated by the decrease in

Fig. 2. Time course for stimulation by 30 μM-ATP[S] in the presence of 1.8 mM-extracellular Ca²⁺

(a) [³²P]PBut; (b) [³²P]PA; □, stimulated; ■, unstimulated controls. Each experiment was in the presence of 50 mM-butanol, and data (means ± S.E.M., *n* = 3) are shown from a single experiment representative of three separate experiments.

Fig. 3. Time course for stimulation by 30 μM-ATP[S] in the presence of 100 nM extracellular Ca²⁺

(a) [³²P]PBut; (b) [³²P]PA; □, stimulated; ■, unstimulated controls. Each experiment was in the presence of 50 mM-butanol, and data (means ± S.E.M., *n* = 3) are shown from a single experiment representative of three separate experiments.

[³²P]PBut production at 30 s (1.32 ± 0.17 versus 2.51 ± 0.16) and 20 min (1.30 ± 0.10 versus 3.21 ± 0.17). The apparently rapid rise in basal value is due to the change of scales (compare Fig. 3a with Fig. 2a), as the basal value at 20 min was 2.33 ± 0.64 times higher

Table 3. Effects of PKC inhibitor Ro 31-8220

Data are expressed as c.p.m. accumulating in PBut or PA (means $\pm$ S.E.M., $n = 3$) with a 3 min incubation from a single experiment representative of three. The control (unstimulated) values from this experiment were [32 P]PBut 1960 ± 190 c.p.m., [32 P]PA 1338 ± 225 c.p.m.

Concn. of Ro 31-8220 (μ M)...	$[^{32}\text{P}]\text{PBut}$ or $[^{32}\text{P}]\text{PA}$ (c.p.m.)			
	0	0.1	1	10
$[^{32}\text{P}]\text{PBut}$				
ATP[S] (30 μ M)	5828 $\pm$ 242	5820 $\pm$ 210	1615 $\pm$ 172	776 $\pm$ 94
PMA (100 nM)	6430 $\pm$ 435	—	2798 $\pm$ 424	681 $\pm$ 68
$[^{32}\text{P}]\text{PA}$				
ATP[S] (30 μ M)	6045 $\pm$ 721	5986 $\pm$ 264	1923 $\pm$ 151	6313 $\pm$ 504
PMA (100 nM)	2064 $\pm$ 63	—	2604 $\pm$ 689	2513 $\pm$ 142

Table 4. Effect of propranolol on [32 P]PA and [32 P]PBut accumulation

Control and stimulated (by 30 μ M-ATP[S]) radioactivity in PA and PBut (with butanol only) in the presence and absence of propranolol. Stimulation was for 3 min; propranolol was present 5 min before, and during, the stimulation. The data are c.p.m. (means $\pm$ S.E.M., $n = 3$) from a single experiment representative of three separate experiments.

	No butanol	50 mM-Butanol	
	[32 P]PA	[32 P]PA	[32 P]But
Control	2389 $\pm$ 524	1823 $\pm$ 178	5090 $\pm$ 314
ATP[S] (30 μ M)	7228 $\pm$ 770	7574 $\pm$ 342	11468 $\pm$ 358
Propranolol (250 μ M)	8087 $\pm$ 625	5629 $\pm$ 186	5324 $\pm$ 274
Propranolol + ATP[S]	20559 $\pm$ 2983	21092 $\pm$ 689	8668 $\pm$ 130

than at 0 min with 100 nM- Ca^{2+} and 2.35 ± 1.11 with 1.8 mM- Ca^{2+} . [32 P]PA accumulation at 30 s was not significantly affected by removal of extracellular Ca^{2+} (1.48 ± 0.15 versus 1.81 ± 0.08), but there was a decrease at 20 min (1.65 ± 0.91 versus 3.89 ± 0.74). The difference in absolute radioactivity (c.p.m.) between Figs. 2(b) and 3(b) is due to variations between experiments unrelated to the levels of extracellular Ca^{2+} .

Stimulation of PLD by PMA suggested a role for PKC, and to investigate this we used the relatively specific inhibitor Ro 31-8220 (e.g. under the same conditions Ro 31-8220 shows 12.5-fold greater selectivity for PKC over cyclic AMP-dependent protein kinase than does staurosporine [21]). Generation of [32 P]PBut was inhibited by the PKC inhibitor during stimulation by ATP[S] or PMA (Table 3). Across three separate experiments (each in triplicate) Ro 31-8220 decreased the ATP[S] (30 μ M) stimulation of [32 P]PBut to $73 \pm 10\%$ (0.1 μ M kinase inhibitor), $19 \pm 16\%$ (1 μ M) and $-10 \pm 11\%$ (10 μ M) of the stimulation seen in the absence of kinase inhibitor. The response to 100 nM-PMA was decreased by the kinase inhibitor to $30 \pm 27\%$ by 1 μ M and $-13 \pm 12\%$ by 10 μ M across the three experiments. The negative percentages in the presence of 10 μ M-Ro 31-8220 are not significantly different from zero, but may be contributed to by inhibition of basal PLD activity. [32 P]PA production was affected in a more complex way: a reproducibly biphasic effect with increasing inhibitor concentration was seen with ATP[S] stimulation (as illustrated in Table 3 and seen in each of three such experiments).

Propranolol at 250 μ M concentration has been reported to inhibit PA phosphohydrolase almost completely [22,23]. In order to determine whether PA generated by either pathway could be converted into DAG, we stimulated cells with 30 μ M-ATP[S] in the presence or absence of propranolol. In some cases we included butanol to distinguish between the PLD pathway ([32 P]PBut) and the PLC/DAG kinase pathway ([32 P]PA), and in other cases the incubations were without butanol, and total [32 P]PA formed by both routes was measured. Table 4 shows results from one of three such experiments. In the absence of butanol, propranolol alone gave a considerable stimulation of labelling in [32 P]PA: over the three separate experiments propranolol alone caused a 3.24 ± 0.60 -fold increase. However, the total [32 P]PA labelling produced by ATP[S] stimulation (no butanol) were also considerably greater in the presence of propranolol (Table 4). Over three separate experiments ATP[S] produced a response above basal, which was 2.58 ± 0.36 -fold greater in the presence of propranolol than in its absence. This effect of propranolol was also seen with [32 P]PA labelling in the presence of butanol. The basal labelling in [32 P]PA was raised 2.50 ± 0.40 -fold, and the ATP[S]-stimulated labelling was raised 2.95 ± 0.28 -fold over the three experiments by propranolol at 250 μ M. However, in the presence of butanol the basal labelling in [32 P]PBut was unaffected, whereas that stimulated by ATP[S] in [32 P]PBut was decreased by about 50% by propranolol. To test for the possibility that propranolol directly inhibits the stimulation of PLD, we used PMA at 100 nM to stimulate [32 P]PBut formation in the presence of 50 μ M-butanol and in the absence and presence of 250 μ M-propranolol. The results from two experiments showed that propranolol inhibited the PMA stimulation of [32 P]PBut by 22% and 23% (significant at $P < 0.05$; Student's t test).

DISCUSSION

In this study we have shown that stimulation, with purinergic agonists in the presence of butanol, of bovine aortic endothelial cells prelabelled for 24 h with [32 P]P_i caused a rapid and significant accumulation of both [32 P]PA and [32 P]PBut. The stimulation of this response by 30 μ M-2MeSATP but not by p[CH₂]₂ppA is consistent with the notion that this response was mediated by the P_{2U}-type receptor that has previously been reported on these cells by this laboratory [24] and others [5,25]. In the present study on AG 4762 cells we found that the pyrimidine UTP produced a [32 P]PA and [32 P]PBut response similar in size to that with 2MeSATP and ATP. In the neuronal PC12 cell line we have shown that stimulation of PLC with UTP has the characteristics of action at a common receptor for both ATP and UTP, which may be called a nucleotide receptor [26].

Martin & Michaelis [12] used pulmonary-artery endothelial cells labelled with [3 H]myristic acid. The myristate label was deliberately chosen because of its preferential incorporation into phosphatidylcholine, which was demonstrated to be a PLD substrate. By using [32 P]P_i loading for 24 h we have labelled the major phospholipids and cytosolic ATP, enabling us to measure PA generated directly by PLD and indirectly by DAG kinase. To separate the two pathways, we used butanol to block effectively [32 P]PA production by PLD by diverting it to [32 P]PBut, and so revealed the putative PLC/DAG kinase contribution. Two pieces of evidence presented in this paper validate our method of demonstrating two pathways. Firstly, the concentration of butanol was shown to be optimal for [32 P]PBut production in dose-response experiments. Secondly, PMA, which activated PLD, massively stimulated [32 P]PBut production, with little stimulation of [32 P]PA production. Additionally, we labelled cells with [32 P]P_i for 2.5 h, during which time cellular ATP became highly labelled, with little incorporation into the lipid fraction

(J. R. Purkiss & M. R. Boarder, unpublished work). Stimulation of these cells in the presence of butanol produced little or no [32 P]PBut, but large [32 P]PA responses were still observed. In separate experiments in our laboratory using cultured adrenal chromaffin cells, we found no evidence of agonist-stimulatable PLD activity: in these studies butanol at 50 μ M had no effect on agonist-stimulated PA production [27], further confirming the specific nature of the effect of butanol on PLD pathways, but not on PLC/DAG kinase pathways.

The relative ability of the agonists to stimulate [32 P]PBut production was the same as that for [32 P]PA, implying that the same receptor was responsible for activation of both pathways. This was confirmed by investigation of the dose-response relationships for both [32 P]PA and [32 P]PBut generation to ATP[S] concentration. Though qualitative changes in [32 P]PA were seen with butanol, the actual losses and gains of radioactivity from PA and in PBut are difficult to quantify, because phosphatidylalcohols are reported to be metabolically stable [14] and will accumulate, whereas PA is biologically labile, with a number of possible fates.

Treatment with 100 nM-PMA for 3 min produced the largest [32 P]PBut response seen in any of our experiments, consistent with the widespread reports of PLD activation by PMA (e.g. [27,28]). However, we also found a small stimulation of [32 P]PA production by PMA in the presence of 50 mM-butanol. This could be due to a residual part of the product of the PLD reaction remaining as PA despite the presence of butanol. However, in rat pheochromocytoma PC12 cells used in our laboratory, a several-fold PMA stimulation of PBut occurs, with no formation of PA [26,27], suggesting that, at least in some cells, butanol can convert all the PLD product into PBut. Furthermore, we have used saturation levels of butanol in this paper. An alternative explanation is the presence of a phorbol ester-stimulated phosphatidylcholine PLC as reported in rat aortic smooth-muscle cells [29].

Investigation of the time course of 30 μ M-ATP[S]-stimulated [32 P]PA and [32 P]PBut production revealed no discernible sequence of PLC and PLD activation. However, the first time point studied was 30 s. This was too long to resolve the order of activation. [32 P]PBut accumulated rapidly over the first 3 min of stimulation, but then continued up to 20 min at a rate which approximately paralleled the rise in basal value from 0 to 20 min. The stability of phosphatidylalcohols would therefore suggest that PLD activity returned to basal after 3 min of stimulation with purinergic agonist. The later sustained elevation of [32 P]PA was more difficult to interpret, because of the biological lability of PA, but with buffering of extracellular Ca^{2+} to 50–100 nM this phase was significantly decreased, suggesting that PA was continuously in flux, generation being dependent on extracellular Ca^{2+} entry. The early phase of [32 P]PA generation appeared to be unaffected by decreasing extracellular Ca^{2+} to approximately the cytosolic level, but this sharply contrasted with [32 P]PBut production, which was virtually abolished under these conditions, suggesting a dependence of PLD activation on Ca^{2+} entry. This is supported by the report of stimulation of [^3H]choline release from aortic endothelial cells by the Ca^{2+} ionophore A23187 [13].

PMA stimulated [32 P]PBut formation, consistent with a role for activation of PKC in stimulation of PLD. The complete inhibition of PMA- and purinergic-receptor-mediated stimulation of PLD by Ro 31-8220 (Table 3) confirmed the key role of PKC in the agonist activation of PLD. Other workers have reported varying inhibition of phorbol ester or agonist stimulation of PLD by the non-specific protein kinase inhibitors staurosporine (e.g. [30]) and H7 [31], and down-regulation of PKC by long-term PMA treatment [13,28]. Possibly through the non-specific nature of these agents and variations in experimental

design, that previous work has produced a bewildering array of complete and partial inhibitions of PLD activation. The compound (no. 3 in [21]) that we have used in this study shows a much improved selectivity for PKC over protein kinase A and Ca^{2+} /calmodulin-dependent protein kinase compared with staurosporine [21]. We have found a similar complete dependency of agonist-stimulated PLD on PKC activity in neuronal PC12 [26] and human neuroblastoma cells (L. Wilkes & M. R. Boarder, unpublished work).

The results discussed in the preceding paragraphs indicate that both Ca^{2+} entry and PKC activation are necessary for the agonist stimulation of PLD. This gives rise to a short-lived production of PA. However, a more sustained and slower phase of PA production exists, and this is via the PLC/DAG kinase routes. The transient nature of agonist-stimulated PLD has been reported previously (e.g. [28]), but the present results show that despite this there may be a sustained PA production. In summary, there are three components to agonist stimulation of PA: (i) an early stimulation of PLC independent of extracellular Ca^{2+} ; (ii) a transient stimulation of PLD dependent on extracellular Ca^{2+} (and PKC); (iii) a sustained stimulation of PLC, dependent on extracellular Ca^{2+} . It would be of considerable interest to know which are the substrate phospholipids, and what are the radiol substitutions, for PA production at each of these three components.

The consequences of PLD and PLC activation and the understanding of the possible role of PA formation in cellular regulation are complicated by the two-way metabolism between PA and DAG. For example, if PA derived from PLD acts as a cellular regulator by conversion into DAG and stimulation of PKC, it is possible that a pool of PA derived from DAG kinase may also regulate DAG levels by the subsequent action of PA phosphohydrolase. Alternatively, the PA derived by DAG kinase action may serve simply as an intermediate in the resynthesis back to phospholipids, or PA may have a cellular regulating function of its own.

In an attempt to provide information relating to these questions, we have used the phosphohydrolase inhibitor propranolol [22,23]. We showed that both the basal and the stimulated labelling in PA is increased by propranolol. This indicates that there is basal turnover of PA by the phosphohydrolase route, and that PA produced by agonist stimulation is, at least in part, converted into DAG. When the experiment was performed in the presence of butanol, the same observation was made: propranolol considerably enhanced the agonist stimulation of [32 P]PA accumulation. In this case the PA originated solely from the sequential action of PLC and DAG kinase. These experiments then show that PA originating from DAG may serve as a precursor pool for subsequent conversion back into DAG. Furthermore, propranolol inhibits the purinergic-receptor-mediated stimulation of PLD in the presence of butanol: this is consistent with a functional role for this DAG originating from the cycle DAG to PA back to DAG.

There have been several reports in the literature supporting the proposition that PA derived from PLD activation serves as a source of DAG (e.g. [32,33]). Here we extend this concept by providing clear evidence that PA synthesized by DAG kinase is recycled back to DAG, and show data suggesting that this DAG plays a role in cellular regulation. The demonstration that a transient elevation of PLD activity may give rise to a sustained formation of DAG from PA [32] suggests that the pathway DAG to PA to DAG may have important consequences on the kinetics of DAG elevation, and hence PKC activation, after agonist stimulation.

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