Phospholipase D-induced phosphatidate production in intact small arteries during noradrenaline stimulation: involvement of both G-protein and tyrosine-phosphorylation-linked pathways

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To investigate membrane lipid metabolism during smooth-muscle activation, the role of phospholipase D (PLD) in the production of phosphatidate (PA) was studied in rat small arteries stimulated with noradrenaline. Incubation with [³H]myristate preferentially labelled phosphatidylcholine (PtdCho), and in the presence of 0.5% ethanol [³H]phosphatidylethanol ([³H]PET) was formed, demonstrating PLD activity. Noradrenaline (NA) stimulation resulted in an increase in PtdCho derived [³H]PA and [³H]PET formation, indicating PLD activation. Stimulation of [¹⁴C]choline release confirmed PLD-mediated hydrolysis of PtdCho. Propranolol, an inhibitor of PA phosphohydrolase, increased [³H]PA levels in non-stimulated tissue and decreased the rate of degradation of both [³H]PA and [³H]PET, implying that this is an active route for PA metabolism in small arteries. However, [³H]diacylglycerol levels were not increased during NA stimulation. Fluoroaluminate increased [³H]PET formation and [¹⁴C]choline release, whereas high K⁺ in the presence of α_1 -

adrenoceptor blockade did not. Pervanadate increased phosphotyrosine levels in small arteries, and markedly stimulated [³H]PET formation and [¹⁴C]choline release. The combination of pervanadate and NA stimulation resulted in a dramatic increase in [³H]PET formation, which was greater than the sum of the individual responses to the two agonists. Pervanadate and fluoroaluminate in combination appeared to give an additive response, whereas high K⁺ did not alter the pervanadate-induced formation of [³H]PET. Phosphotyrosine levels were increased by NA in the presence of tyrosine phosphatase inhibitors. This effect was blocked by genistein, a tyrosine kinase inhibitor. These data demonstrate that in NA-stimulated small arteries PLD-induced PtdCho hydrolysis contributes to accumulation of PA, but not of diacylglycerol. Furthermore, regulation of PLD activity appears to require G-protein and tyrosine-phosphorylation-linked pathways.

INTRODUCTION

Stimulation of rat small arteries with noradrenaline (NA) results in the rapid production of inositol trisphosphate through activation of phosphoinositide-specific phospholipase C (PI-PLC) [1]. Diacylglycerol (DAG), the second product of inositol lipid hydrolysis, appears to accumulate more slowly, due to its rapid metabolism to phosphatidic acid (PA) by the action of DAG kinase [2]. Although NA stimulation generates PA enriched with arachidonate, indicating an origin from inositol lipids [2], it is also possible that an additional source of PA may be phosphatidylcholine (PtdCho) hydrolysis. Indeed, it has been demonstrated that stimulation of vascular smooth-muscle preparations with NA results in the hydrolysis of PtdCho by phospholipase D (PLD) [3–5]. Furthermore, in the rat aorta, the time course for changes in PA was closely related to Ca²⁺-dependent tonic contraction, suggesting that PA may be important for the regulation of Ca²⁺ entry during NA-stimulated vasoconstriction [5]. However, PA generated by PLD-mediated PtdCho hydrolysis may itself be dephosphorylated to DAG, leading to protein kinase C (PKC) activation [6,7], a pathway which may be involved in smooth-muscle contraction also [8].

Detailed studies using various cell types have demonstrated PLD hydrolysis of PtdCho in response to receptor stimulation (reviewed in [6,7]), which in most cases are coupled to G-proteins. However, the mechanisms by which diverse agonists stimulate PLD activity remain unclear. Biochemical and pharmacological studies in a variety of cells or tissues implicate PKC

[9–16] or G-proteins [17–19], although in rat aorta Jones and co-workers [5] have shown that NA-stimulated PLD activity is independent of PKC or Ca²⁺ influx. More recently, stimulation of tyrosine kinase receptors or inhibition of tyrosine phosphatases by agents such as vanadate has implicated tyrosine phosphorylation in PLD activation [20–23]. Furthermore, Dubyak and co-workers [24] observed a synergistic activation of PLD by G-protein and tyrosine kinase-based mechanisms in permeabilized phagocytic leucocytes.

In this study we have investigated the regulation of PLD activity during NA-induced contraction of intact rat small arteries. NA stimulation leads to PLD-mediated PtdCho hydrolysis, with the subsequent accumulation of PA but not DAG. In addition, we have identified a link between G-proteins and tyrosine phosphorylation in the regulation of PLD activity, and provide evidence that NA activates tyrosine kinases. These results suggest that PLD activity is regulated by multiple signalling pathways in intact small arteries.

EXPERIMENTAL

Materials

Radioisotopes, [³H]myristic acid (specific radioactivity 33.5 mCi/mmol) and [¹⁴C]choline chloride (specific radioactivity 53 mCi/mmol) were purchased from DuPont-NEN. Ethanol was from Hayman Ltd. Tissue-culture medium (M199) was from Gibco-BRL, Paisley, Scotland, U.K. Phosphatidylethanol (PET) was

Abbreviations used: NA, noradrenaline; PI-PLC, phosphoinositide-specific phospholipase C; DAG, diacylglycerol; PA, phosphatidate; PtdCho, phosphatidylcholine; PLD, phospholipase D; PAP, PA phosphohydrolase; PET, phosphatidylethanol; PKC, protein kinase C; PV, pervanadate; AlF₄⁻, fluoroaluminate.

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supplied by Avanti Polar Lipids, Birmingham, AL, U.S.A. TLC plates (Merck 5721) and 'HiPerSolv' grade solvents were purchased from BDH, Poole, Dorset, U.K. Scintillation fluid (Ultima Gold MV) was from Canberra Packard, Pangbourne, Berks, U.K. Protogel [30% (w/v) acrylamide] was from National Diagnostics (Lancs, U.K.). Hybond-C extra nitrocellulose paper and Enhanced Chemiluminescence (ECL) kits were from Amersham International, U.K. Anti-phosphotyrosine antibody (coupled to horseradish peroxidase) was purchased from Transduction Laboratories (Nottingham, U.K.). All other chemicals were purchased from Sigma (U.K.).

Preparation of small arteries

Adult Sprague-Dawley rats (200–300 g) were killed by stunning and cervical dislocation. The mesentery was immediately excised and kept in ice-cold physiological salt solution [(mM) 119 NaCl, 4.7 KCl, 25 NaHCO₃, 1.17 MgSO₄·7H₂O, 1.18 KH₂PO₄, 0.026 K₂EDTA, 5.5 glucose, 2.5 CaCl₂·2H₂O, pH 7.2] until dissection. Mesenteric small arteries of < 300 µm internal diam. were cleaned of adjoining fat and connective tissues before use in all subsequent stages.

Assay of PLD transphosphatidylase activity

A 10 µCi portion of [³H]myristic acid was dried under a stream of nitrogen and redissolved in 200 µl of tissue-culture medium (M199) by bath sonication for 5 min. Vessels were incubated in the [³H]myristate/M199 solution for 3 h in a 37 °C water bath, washed in 3 ml of M199 for 15 min and transferred to 3 ml of fresh M199 with or without 0.5% ethanol and with or without 10 µM NA for a further 20 min at 37 °C. After stimulation, lipids were extracted as previously described [2]. Briefly, 20 µl of 10 mg/ml PA/PEt was added to vessels which were first homogenized on ice in 0.5 ml of chloroform/methanol/HCl (20:40:1, by vol.). The homogenate was left on ice for 10 min; 0.5 ml of chloroform and 0.5 ml of water were added, mixed, and the mixture was spun at 12000 g for 5 min. The lower organic phase was transferred to a glass vial, evaporated to dryness under a stream of nitrogen gas and redissolved in 30 µl of chloroform. The residual protein pellet was dissolved in 2 M NaOH and the protein content was determined [25].

[³H]PA and [³H]PEt were separated from other ³H-containing components by one-dimensional TLC on 0.25 mm-thick oxalate-coated silica gel 60 plates. Ethyl acetate/acetic acid/trimethylpentane (9:2:5, by vol.) was used as developing solvent [2]. Bands corresponding to PA and PEt, revealed by iodine staining and identified from co-spotted authentic standards, were scraped from the plate into mini scintillation vials, mixed with 3 ml of scintillation fluid and counted for ³H radioactivity.

For the measurement of [³H]DAG levels, lipid extracts were spotted on silica gel 60 plates and developed in light petroleum (b.p. 40–60 °C)/diethyl ether/acetic acid (200:67:5, by vol.). The band corresponding to authentic 1,2-DAG standard was scraped from the plate and quantified by liquid-scintillation counting.

The incorporation of [³H]myristate into individual phospholipids was determined by incubating three sets of vessels in the [³H]myristate/M199 solution for 1, 2 and 3 h respectively. Phospholipids were extracted as detailed above and resolved by TLC using a chloroform/methanol/methylamine (30:10:3, by vol.) solvent mixture. Bands corresponding to authentic phospholipid standards were scraped from the plate and quantified by liquid-scintillation counting. Data were calculated as a percentage of total ³H in the phospholipids.

[¹⁴C]Choline release assay

[¹⁴C]Choline chloride (5 µCi) was dried under a stream of N₂, redissolved in 200 µl of M199 and sonicated for 5 min. Vessels were incubated in [¹⁴C]choline/M199 solution for 3 h at 37 °C, washed in 3 ml of M199 for 5 min, washed again for 20 min and transferred to 1.1 ml of M199 for a 20 min incubation with or without 10 µM NA. Vessels were removed from the medium, homogenized as described above, and the particulate protein phase was air-dried and dissolved in 0.5 ml of 2 M NaOH before protein estimation [25]. [¹⁴C]Choline was extracted from the remaining medium by the method of Kennerly [26]. Briefly, 0.765 ml of 12 mM sodium phosphate (pH 7.0) was added to 1 ml of medium from the final vessel incubation and mixed. Choline was then extracted into the upper heptanone phase generated by adding 1.178 ml of 4 mg/ml tetraphenylboron in heptan-4-one. After vigorous mixing and phase separation by centrifugation, 1.06 ml of the heptanone phase was removed, mixed with 0.353 ml of 1 M HCl and spun in a Microcentaur centrifuge. Then 300 µl of the lower, choline-containing aqueous phase was transferred to a scintillation vial containing 3 ml of scintillant and quantified by liquid-scintillation counting. No contamination of the [¹⁴C]choline extract with [¹⁴C]phosphocholine was found if the extract was separated by TLC (results not shown). Data were expressed as d.p.m./mg of protein.

Phosphotyrosine immunoblots

Vessels were equilibrated at 37 °C in 1 ml of M199 and transferred to pre-warmed 1 ml portions of M199 with or without 300 µM pervanadate (PV) for up to 20 min. Vessels were homogenized in 150 µl of ice-cold homogenization buffer [(mM) 20 Tris, 250 sucrose, 5 EDTA, 10 EGTA, 10 dithiothreitol, pH 7.5] with the following additions: 1 mM sodium orthovanadate, 200 µM sodium pyrophosphate, 50 mM NaF, 1 mM phenylmethanesulphonyl fluoride and 100 µM leupeptin. A sample was removed for protein estimation. The remaining homogenate was mixed with 5-fold-concentrated Laemmli sample buffer, heated at 100 °C for 3 min and resolved by SDS/PAGE. After electrophoresis, proteins were electrophoretically transferred to Hybond-C by the method of Towbin et al. [27]. Membranes were processed for Western-blot analysis according to the manufacturer's recommended protocol. The primary antibody was anti-phosphotyrosine antibody coupled to horseradish peroxidase. Signals were developed by using an ECL kit (Amersham).

Protein was estimated by the Lowry method [25].

Statistical analyses

Results are expressed as means ± S.E.M., and comparisons between basal and agonist-induced changes were made by Student's *t* test.

RESULTS

NA-stimulated hydrolysis of PtdCho

Incubation of rat mesenteric small arteries with [³H]myristate resulted in 60 ± 4% of the total radioactivity in phospholipids being preferentially incorporated in PtdCho, with only 3 ± 1.8% in PtdIns and PA after 3 h. The values of radioactivity at 3 h were: total phospholipids 1526684 ± 406054, PtdCho 913493 ± 238529 and PtdIns + PA 37853 ± 1844 d.p.m./mg of protein

Figure 1 Effect of NA stimulation on $[^3\text{H}]\text{PA}$ and $[^3\text{H}]\text{DAG}$ levels in rat small arteries

$[^3\text{H}]\text{Myristate}$ -labelled mesenteric arteries were stimulated with NA ($10\ \mu\text{M}$) for various time points up to 20 min. The lipids were extracted and quantified as described in the Experimental section. (a) $[^3\text{H}]\text{PA}$; (b) $[^3\text{H}]\text{DAG}$. The results are means $\pm$ S.E.M. from a minimum of 3 separate experiments: * $P < 0.01$ by Student's t test. Dotted line indicates mean control value. The mean basal radioactivity was: $[^3\text{H}]\text{phospholipids}$ 2588737 ± 550161 , $[^3\text{H}]\text{PA}$ 17440 ± 4140 , $[^3\text{H}]\text{DAG}$ 281640 ± 50481 d.p.m./mg of protein.

($n = 3$). This is in good agreement with other studies using intact vascular tissue [3,5].

The stimulation of myristate-labelled small arteries with NA ($10\ \mu\text{M}$) resulted in an increase in $[^3\text{H}]\text{PA}$ levels (+50% at 5 min). The levels of this lipid then returned towards baseline at 20 min (Figure 1a). In a separate set of experiments where PtdCho was labelled with $[^{14}\text{C}]\text{choline}$, NA stimulation of vessels resulted in an increased release of $[^{14}\text{C}]\text{choline}$ at the same time points as $[^3\text{H}]\text{PA}$ production (Figure 1a, Table 1). $[^{14}\text{C}]\text{Choline}$ release was inhibited by prazosin ($10\ \mu\text{M}$), indicating that NA was acting through α_1 -adrenoceptors. In contrast, $[^3\text{H}]\text{DAG}$ levels remained constant for up to 20 min of NA ($10\ \mu\text{M}$) stimulation (Figure 1b). These results suggest that NA transiently activates PLD-mediated hydrolysis of PtdCho .

To measure PLD activity directly, we made use of a transphosphatidyl reaction unique to the enzyme, in which the phospholipid headgroup is exchanged for ethanol, producing PEt instead of PA . In the presence of 0.5% ethanol, NA stimulation of $[^3\text{H}]\text{myristate}$ -labelled vessels resulted in a rapid but transient rise in $[^3\text{H}]\text{PEt}$ formation. The increase was apparent after 20 s

Table 1 Effect of NA on release of $[^{14}\text{C}]\text{choline}$ from prelabelled rat small arteries

$[^{14}\text{C}]\text{Choline}$ -labelled vessels were stimulated with $10\ \mu\text{M}$ NA for various times up to 20 min. A separate set of samples was incubated with $10\ \mu\text{M}$ prazosin for 15 min and then co-incubated with $10\ \mu\text{M}$ NA for 5 min. $[^{14}\text{C}]\text{Choline}$ was extracted from the medium as described in the Experimental section. Results are expressed as the percentage increase in $[^{14}\text{C}]\text{choline}$ release above control, and are means $\pm$ S.E.M. of a minimum of three separate experiments. Basal $[^{14}\text{C}]\text{choline}$ release was 2666 ± 354 d.p.m./mg of protein ($n = 4$).

Treatment	Time (min)	$[^{14}\text{C}]\text{Choline}$ release (% change from control)
NA	0.33	37 ± 19
NA	5	118 ± 27
NA	20	74 ± 11
NA + prazosin ($10\ \mu\text{M}$)	5	15 ± 10

Figure 2 Effect of noradrenaline stimulation on $[^3\text{H}]\text{PEt}$ levels in rat small arteries

$[^3\text{H}]\text{Myristate}$ -labelled mesenteric arteries were stimulated with NA ($10\ \mu\text{M}$) in the presence of 0.5% ethanol for various times up to 20 min. The lipids were extracted and quantified as described in the Experimental section. The results are means $\pm$ S.E.M. from a minimum of 3 separate experiments: * $P < 0.01$, ** $P < 0.001$, by Student's t test. The mean basal radioactivity was: $[^3\text{H}]\text{phospholipids}$ 2149462 ± 245151 , $[^3\text{H}]\text{PEt}$ 2145 ± 268 d.p.m./mg of protein.

(+73%, $P < 0.01$) and further increased at 2.5 min (+94%, $P < 0.01$). Subsequently $[^3\text{H}]\text{PEt}$ levels stayed slightly above control during the remaining 20 min of NA stimulation (Figure 2). These results demonstrate a rapid stimulation of PLD activity in intact small arteries by NA. If PEt were metabolically stable [28], the plateau phase of $[^3\text{H}]\text{PEt}$ formation in response to NA (Figure 2) would indicate transient PLD activation. However, we have previously shown that PA is rapidly metabolized in rat small arteries [29], and, to determine whether PEt behaved in a similar manner, we undertook the investigation of $[^3\text{H}]\text{PEt}$ and $[^3\text{H}]\text{PA}$ metabolism. Basal levels of $[^3\text{H}]\text{PEt}$ and $[^3\text{H}]\text{PA}$ were elevated by stimulating $[^3\text{H}]\text{myristate}$ -labelled vessels with $300\ \mu\text{M}$ PV and $10\ \mu\text{M}$ NA in the presence of 0.5% ethanol for 20 min (the radioactivity in $[^3\text{H}]\text{PEt}$ and $[^3\text{H}]\text{PA}$ was increased to 35750 and 77021 d.p.m./mg of protein respectively by this treatment; Table 2). $[^3\text{H}]\text{PA}$ and $[^3\text{H}]\text{PEt}$ were subsequently measured as a function of time. Levels of both lipids fell rapidly during the first 1 h after stimulation to approx. 50% of zero-time values, but did not decrease further during the second hour

Table 2 Time course of [^3H]PA and [^3H]PE metabolism after co-stimulation with PV and NA

Vessels prelabelled with [^3H]myristic acid for 3 h were incubated for 20 min in the presence of 300 μM PV and 10 μM NA. They were then incubated in agonist-free medium, and samples were removed at various time points up to 2 h after stimulation. One set of samples was incubated in 1 mM propranolol for 60 min after the PV/NA stimulation. Lipids were extracted and quantified as described in the Experimental section. Results are expressed as a percentage of total [^3H]labelled phospholipids, and are means $\pm$ S.E.M. of three experiments. Mean radioactivity values at zero time were [^3H]phospholipids 3039927 ± 852376 , [^3H]PA 77021 ± 24305 and [^3H]PEt 35750 ± 8791 d.p.m./mg of protein.

Treatment	Time after stimulation (min)	[^3H]PEt	[^3H]PA
	0	1.22 ± 0.08	2.49 ± 0.22
	30	0.90 ± 0.04	1.87 ± 0.13
	60	0.51 ± 0.02	1.44 ± 0.13
	120	0.51 ± 0.13	1.41 ± 0.05
	60	0.98 ± 0.08	16.14 ± 0.39
1 mM propranolol			

(Table 2). In the presence of 1 mM propranolol, an inhibitor of PA phosphohydrolase (PAP) [30–32], the rate of degradation of [^3H]PEt was markedly decreased, with levels falling only 19% in the presence of propranolol, compared with 58% in its absence in the first 1 h after stimulation (Table 2). In contrast, there was a dramatic increase in the levels of [^3H]PA, to 650% above control in the presence of propranolol (Table 2). In addition, [^3H]myristate-labelled vessels in the presence of 1 mM propranolol exhibited [^3H]PA levels of $4.79 \pm 0.31\%$, compared with the basal value of $1.12 \pm 0.19\%$ of total [^3H]phospholipids ($n = 5$; $P < 0.001$). These data imply that both PA and PEt are rapidly metabolized by PAP in small arteries, and are in good agreement with those reported in intact pancreatic islets [33], but not in rat aorta, where PEt was found to be metabolically stable for 3 h even though PA was degraded [5]. High concentrations of propranolol ($> 100 \mu\text{M}$) have been shown to inhibit PKC in human neutrophils [34], which may result in PLD activation [12]. Therefore it is possible that, rather than slowing PA and PEt metabolism through inhibition of PAP, propranolol may have increased the production of these lipids as a result of PLD activation. To investigate such a possibility, [^{14}C]choline release as an indicator of PLD induced PtdCho breakdown was measured in the presence of 1 mM propranolol. There was no change in [^{14}C]choline release over a 20 min period; basal release 11219 ± 2632 d.p.m./mg of protein, 1 mM propranolol 11430 ± 2112 d.p.m./mg of protein. These data would suggest that, rather than activating PLD, propranolol may be inhibiting PAP, an enzyme regulating PA levels in small arteries.

Mechanisms involved in the regulation of PLD in intact small arteries

Receptor-mediated agonist stimulation in a variety of cells and tissues has implicated G-protein and tyrosine kinase-based mechanisms in the regulation of PLD activity [17–24]. Fluoroaluminate (AlF_4^-) is a non-specific G protein activator [35], which causes force development accompanied by membrane depolarization and Ca^{2+} influx in rat mesenteric arteries [36]. Incubation of [^3H]myristate-labelled vessels with AlF_4^- in the presence of 0.5% ethanol resulted in a 73% increase in [^3H]PEt (Table 3). In addition, a similar increase in [^{14}C]choline release ($+81 \pm 31\%$; basal [^{14}C]choline release 11219 ± 2632 d.p.m./mg of protein) was observed after stimulation with the same agonist. These data suggest G-protein-linked PLD activation. To de-

Table 3 Effects of PV, AlF_4^- , NA and 125 mM K^+ on [^3H]PEt levels in rat small arteries

Pre-labelled vessels were stimulated, in the presence of 0.5% ethanol $\pm$ 300 μM PV, with 10 μM AlF_4^- , 10 μM NA or 125 mM KCl + 10 μM prazosin for 20 min. [^3H]PEt was extracted and quantified as described in the Experimental section. Results are expressed as a percentage of [^3H]labelled phospholipids and represent the means $\pm$ S.E.M. of a minimum of three separate experiments: * $P < 0.01$ ** $P < 0.001$ compared with basal. The mean basal radioactivity was [^3H]phospholipids 4137774 ± 541385 , [^3H]PEt 10832 ± 1408 d.p.m./mg of protein.

Treatment	PLD activity ([^3H]PEt as % of total)
Basal	0.265 ± 0.035
PV	$0.675 \pm 0.165^{**}$
AlF_4^-	$0.458 \pm 0.114^*$
PV + AlF_4^-	$0.931 \pm 0.241^{**}$
NA	$0.396 \pm 0.094^{**}$
PV + NA	$1.881 \pm 0.394^{**}$
K^+	0.187 ± 0.028
PV + K^+	$0.587 \pm 0.098^{**}$

Figure 3 Effect of PV on phosphotyrosine levels in rat small arteries as (a) a function of PV concentration after 5 min stimulation, and (b) a function of time at 300 μM PV

The 10 and 20 min time points were exposed to the autoradiograph more briefly than those of 0–2.5 min, due to the large increase in phosphotyrosine levels at the later time points.

termine whether changes in membrane potential and increases in intracellular Ca^{2+} alone were involved in PLD activation, we stimulated vessels with KCl (125 mM) in the presence of prazosin (10 μM) to block the effects of neuronally released catecholamines. There was no change in [^3H]PEt levels after 20 min stimulation with KCl + prazosin compared with control values (Table 3).

PV, a tyrosine phosphatase inhibitor, increases PLD activity in human neutrophils [37]. There was a time- and dose-related increase in phosphotyrosine levels in small arteries (Figure 3) after incubation with PV (3–300 μM). An increase in phosphotyrosine levels was detected as early as 2.5 min after stimulation, and the levels continued to rise up to 20 min (Figure 3). The increase in phosphotyrosine levels correlated with increased formation of [^3H]PEt ($+155 \pm 62\%$) as well as increased release of [^{14}C]choline ($+67 \pm 33\%$; basal [^{14}C]choline release 11219 ± 2632 d.p.m./mg of protein) at 20 min of PV (300 μM) treatment (Table 3), suggesting that PLD-mediated hydrolysis of PtdCho involves tyrosine phosphorylation.

Recent evidence demonstrates that agonists which act through G-protein-linked receptors and the phosphoinositide signalling pathway also stimulate tyrosine kinases in vascular smooth-

Figure 4 Effect of NA on phosphotyrosine levels in rat small arteries

(a) Effect of 20 min stimulation with 10 μ M NA (lane 2) and 300 μ M PV (lane 3). Lane 1, basal. (b) Effect of 10 μ M NA (lane 2) on phosphotyrosine recovery during 15 min incubation after a 10 min stimulation with 30 μ M PV (lane 1); 100 μ M genistein (lane 3) (tyrosine kinase inhibitor) was used to block the response.

muscle cells [38–40]. Therefore we investigated the effects of combined G-protein and phosphotyrosine stimulation on PLD activity. AlF_4^- (10 μ M) and PV (300 μ M) increased [^3H]PEt formation to levels approximately equal to the sum of their individual effects (AlF_4^- + 71 %, PV + 155 %, AlF_4^- + PV + 251 %) (Table 3), whereas KCl (125 mM) + prazosin (10 μ M) and PV increased [^3H]PEt levels to the same extent as PV alone (PV + 155 %, KCl + PV + 122 %) (Table 3). In contrast, simultaneous stimulation with NA (10 μ M) and PV (300 μ M) caused a dramatic increase in [^3H]PEt formation (+ 610 %), which was 2.3-fold greater than the sum of the effects of the individual treatments. There was also a greater release of [^{14}C]choline when these two treatments were applied together (NA + 74 %, PV + 67 %, NA + PV + 152 %; basal [^{14}C]choline release 11219 ± 2632 d.p.m./mg of protein).

Finally, we investigated the effects of NA on phosphotyrosine levels in intact small arteries. Compared with controls, there was no change in protein tyrosine phosphorylation after 20 min stimulation with 10 μ M NA (Figure 4a). However, after 10 min pretreatment with PV (30 μ M), NA stimulation increased phosphotyrosine levels, an effect attenuated by the tyrosine kinase inhibitor genistein (Figure 4b) [41]. These data suggest that NA activates tyrosine kinases.

DISCUSSION

Although it is clear that inositol trisphosphate-mediated discharge of stored Ca^{2+} occurs sufficiently rapidly to initiate contraction, the intracellular events that sustain this process are uncertain. The time course for NA-stimulated PA production in small arteries is consistent with it having a role in the contractile response. An increase in PA levels was detected early and was sustained for at least 5 min. Previously we have shown that PA is formed during NA stimulation from arachidonate-containing DAG produced during PI-PLC hydrolysis of inositol lipids [2]. In this study we have demonstrated that PtdCho is also a substrate for PLD during NA stimulation. The activation of PLD by NA was rapid, but appeared to be transient, as levels of [^3H]PEt did not increase further after 2.5 min of stimulation. But this interpretation assumes that PEt is metabolically stable: indeed, it has been demonstrated in neutrophils, as well as in rat aorta, that there is minimal degradation of PEt up to 3 h [5,28]. However, this assumption may not be valid in intact small

arteries, because both [^3H]PA and [^3H]PEt were rapidly metabolized; over 1 h the levels of both of these lipids fell to less than 50 % of the initial values. When vessels were labelled with [^{14}C]choline, the release of the headgroup into the surrounding medium correlated with both [^3H]PA and [^3H]PEt production after NA stimulation, confirming that PtdCho was the substrate for PLD. Again the response appeared to be transient. Previous studies of PtdCho hydrolysis in intact vascular tissues have demonstrated sustained responses to NA. For instance, in the rat aorta two studies have demonstrated PtdCho hydrolysis at 30 min [5] and 60 min [42] after NA. In the rat tail artery PtdCho breakdown was reported after 10 s and up to 30 min of NA stimulation [3]. Therefore the transient effect in rat mesenteric arteries indicates a difference in the response to NA between large and small vessels, possibly reflecting differences in their physiological roles.

It has been suggested that PA formed by PLD hydrolysis of PtdCho is metabolized to 1,2-DAG, resulting in sustained production of the latter and therefore maintained activation of PKC [6]. PAP catalyses the conversion of PA into DAG, and a plasma-membrane-bound PAP has been characterized and proposed to have a role in signal transduction [30,43,44]. The recently identified property of propranolol to inhibit selectively the conversion of PA into DAG by PAP [30,45] has been used to examine the role of PA-derived DAG in cellular signalling processes. Propranolol markedly attenuated the degradation of [^3H]PEt and [^3H]PA and raised basal levels of [^3H]PA, implying that PAP is involved in regulating PA levels. However, a recent report that propranolol also inhibits PKC raises doubts as to its specificity [34]. Therefore we checked further whether propranolol directly affected PLD activity. After labelling with [^{14}C]choline, propranolol did not increase the release of [^{14}C]choline into the surrounding medium, demonstrating that PtdCho hydrolysis was not stimulated. Therefore the increased basal levels of PA in the presence of propranolol must be attributable to decreased metabolism.

Even though we were able to demonstrate an active PAP pathway in small arteries, [^3H]DAG did not accumulate during NA stimulation. This finding is in agreement with our previous study where we were unable to detect an increase in total 1,2-DAG levels after NA stimulation [2], presumably due to its metabolism by a DAG lipase and/or kinase, the balance of which may alter during receptor stimulation. Certainly NA stimulation of rat small arteries rapidly activates a membrane-associated DAG kinase [46]. Metabolism of PtdCho-derived 1,2-DAG by this pathway may contribute to the large increase in [^3H]PA compared with [^3H]PEt observed in the presence of propranolol. Further studies are required to establish the route of PtdCho-derived DAG metabolism.

Regulation of PLD activity

In the second part of the study we investigated two mechanisms which may be involved in controlling PLD activity in small arteries. We used AlF_4^- to activate G-proteins, and observed an increase in PLD activity and PtdCho hydrolysis. This effect was not solely due to membrane depolarization and Ca^{2+} influx, as high K^+ did not affect enzyme activity. In agreement with findings in other systems [47,48], our results imply a direct link between G-protein activation and PLD.

There is considerable evidence that tyrosine phosphorylation is involved in the regulation of PLD activity [20–23], and recent reports show that agonists which activate PI-PLC can also stimulate tyrosine phosphorylation in cultured vascular smooth-muscle cells [38–40]. PV, a tyrosine phosphatase inhibitor [22],

induced a large increase in protein tyrosine phosphorylation, suggesting that phosphotyrosine phosphatase activity is high in resting small arteries. In addition, PV stimulated PLD activity and PtdCho breakdown, suggesting that tyrosine phosphorylation may be involved in the agonist-stimulated regulation of PLD activity. PV and AlF_4^- appear to activate PLD by different pathways, as their responses were additive. The increase in PV-stimulated PLD activity in the presence of AlF_4^- was not due solely to raised intracellular Ca^{2+} , as high K^+ did not increase the PV response. Surprisingly, PV and NA caused a dramatic increase in PtdCho hydrolysis, which was 2.3-fold greater than the sum of the response to the individual agonists. This synergistic effect implies that phosphotyrosine phosphatase may have an important inhibitory role, attenuating the activation of PLD during NA stimulation. Indeed, it was only after vessel pretreatment with PV that we could detect enhanced phosphotyrosine levels, an effect attenuated by tyrosine kinase inhibition. These data indicate that NA stimulates tyrosine kinase activity, an effect masked by phosphatases, suggesting that a balance between tyrosine kinase and phosphatase activity may play a crucial role in lipid second-messenger generation during receptor activation. Recently, Di Salvo and co-workers [49] have shown that tyrphostin, a tyrosine kinase inhibitor [50], reversibly blocked the contractile response of guinea pig mesenteric microvessels to NA, but did not affect the tension development in response to K^+ , a non-receptor-mediated response. Their data suggest that tyrosine phosphorylation may be involved in agonist-stimulated smooth-muscle contraction.

The data presented in this study demonstrate that PLD activity is regulated by at least two distinct pathways in small arteries. Furthermore, during NA-induced vasoconstriction, activation of PLD and the subsequent hydrolysis of PtdCho contribute to the formation of PA, a potential second messenger. Therefore, it is important that further detailed studies be undertaken to characterize the PLD forms, their substrate preferences and mechanisms of activation in intact vascular smooth muscle.

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