Evidence for receptor and G-protein regulation of a phosphatidylethanolamine-hydrolysing phospholipase A₁ in guinea-pig heart microsomes: stimulation of phospholipase A₁ activity by DL-isoprenaline and guanine nucleotides

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While evidence has been presented for the receptor-mediated activation of phospholipases A₂, C and D, the activation of phospholipase A₁ subsequent to receptor activation has not been established. Phospholipase A₁-catalysed hydrolysis of 1-palmitoyl-2-linoleoyl-glycerophosphoethanolamine (GPE) by guinea-pig heart microsomes was stimulated 40–60% by isoprenaline. This isoprenaline-mediated increase in activity was blocked by propranolol and butoxamine, a specific β_2 -adrenergic antagonist, but not by atenolol, a specific β_1 -adrenergic antagonist. Neither clonidine nor phenylephrine, α_1 - and α_2 -adrenergic agonists respectively, had a stimulatory effect on the hydrolysis of the PE substrate. Guanosine 5'-[γ -thio]triphosphate (GTP[S]) and guanosine 5'-[β,γ -imido]triphosphate, but not

guanosine 5'-[β -thio]diphosphate (GDP[S]) or adenosine 5'-[γ -thio]triphosphate, stimulated the hydrolysis of 1-palmitoyl-2-linoleoyl-GPE by phospholipase A₁. GDP[S] inhibited the isoprenaline-mediated stimulation of phospholipase A₁ activity. Phospholipase A₁ hydrolysis of 1-palmitoyl-2-linoleoyl-GPE was not dependent on cations; however, the stimulatory effects of isoprenaline and GTP[S] on the hydrolytic activity were abolished by cation chelators. The above data suggest that phospholipase A₁ activity in guinea-pig heart microsomes is activated by the binding of isoprenaline to β_2 -adrenergic receptors. Furthermore the stimulation of phospholipase A₁ activity by the agonist may be mediated via activation of G-proteins.

INTRODUCTION

The binding of agonists to cell surface receptors usually initiates processes that result in characteristic physiological responses to the ligand. There are several mechanisms by which the signals from membrane receptors are transduced to the interior of the cell. One mechanism involves the hydrolysis of phospholipids to produce molecules that participate in processes that lead to cell activation [1]. A well established example of this is the hydrolysis of inositol lipids by phospholipase C subsequent to cell stimulation [2]. Agonist-induced hydrolysis of other membrane phospholipids, particularly the choline phospholipids, by phospholipases A₂, C and D is also thought to play an important role in the activation of cellular processes [3–5]. Only phospholipases of the A₁ type have not been shown to respond to activation of receptors. Evidence that phospholipases A₁ may be modulated by external agonists is suggested in a study by Franson et al. [6]. While these authors did not discriminate between phospholipases A₁ and A₂, their results led to the conclusion that canine heart phospholipase A activity, a component of which is phospholipase A₁, was activated by DL-isoprenaline. The mechanism underlying the activation was not examined [6]. In this study we provide evidence that the activity of a phosphatidylethanolamine (PE)-hydrolysing phospholipase A₁ in guinea-pig heart microsomes is enhanced by DL-isoprenaline acting via β -adrenergic receptors and G-proteins.

EXPERIMENTAL

Materials

1-Stearoyl-2-[1-¹⁴C]arachidonoyl-GPE (GPE is *sn*-glycero-3-phosphoethanolamine) (55 mCi/mmol), 1-palmitoyl-2-[1-¹⁴C]linoleoyl-GPE (55 mCi/mmol) and L-lyso-1-[1-¹⁴C]palmitoyl-3-phosphatidylcholine (50 mCi/mmol) were purchased from Amersham International (Oakville, Ontario, Canada). 1-Palmitoyl-2-linoleoyl-GPE, 1-stearoyl-2-arachidonoyl-GPE, DL-isoprenaline, 1-palmitoyl-2-linoleoyl-GPC (GPC is *sn*-glycero-3-phosphocholine), DL-propranolol, butoxamine, clonidine, phenylephrine, phospholipase A₂ (*Crotalus adamanteus*) and sodium taurodeoxycholate were purchased from the Sigma Chemical Co. (St. Louis, MO, U.S.A.). Guinea pigs were acquired from Charles River (St. Constant, Quebec, Canada). Pig liver lyso-PE was bought from Serdary Research Laboratories (London, Ontario, Canada). Ecolite was purchased from ICN Biomedicals (St. Laurent, Quebec, Canada). All nucleotides were obtained from Boehringer Mannheim (St. Laval, Quebec, Canada). All other solvents and chemicals were of reagent grade and were obtained from Baxter-Canlab (Winnipeg, Manitoba, Canada).

Preparation of microsomes

Guinea pigs (250–300 g) were killed and the hearts were removed

Abbreviations used: GPE, *sn*-glycero-3-phosphoethanolamine; PE, phosphatidylethanolamine; GPC, *sn*-glycero-3-phosphocholine; GTP[S], guanosine 5'-[γ -thio]triphosphate; p[NH]ppG, guanosine 5'-[β,γ -imido]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; ATP[S], adenosine 5'-[γ -thio]triphosphate

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and placed on ice. Microsomes were prepared by differential centrifugation of heart homogenates as previously described [7]. Between six and eight guinea-pig hearts were combined for each microsomal preparation. The protein content was determined by the method of Lowry et al. [8] and the subcellular fractions were stored at -80°C .

Preparation of substrates for phospholipase A_1 assays

PE substrates used in phospholipase A_1 assays were prepared by the addition of $0.5\text{ }\mu\text{mol}$ of the PE to a test tube. The solvent was evaporated under a stream of N_2 followed by the addition of $20\text{ }\mu\text{l}$ of sodium taurodeoxycholate (5%, w/v) to give 1 mg of detergent/ $0.5\text{ }\mu\text{mol}$ of PE. Double-distilled water was added to bring the final volume to 1 ml and the mixture was vortex-mixed for 30–60 s.

Phospholipase A_1 assay

Phospholipase A_1 activity was determined by measuring the rate of formation of radiolabelled lyso-PE from 1-acyl-2-[^{14}C]acyl-GPE. The assay mixture contained 100 mM Tris/HCl, pH 9.0, $200\text{ }\mu\text{M}$ PE substrate (specific radioactivity $0.5\text{ }\mu\text{Ci}/\mu\text{mol}$) and $100\text{ }\mu\text{g}$ of guinea-pig heart microsomal protein in a total volume of $500\text{ }\mu\text{l}$. Microsomes were omitted from blank tubes and the results from these were used to correct for non-enzymic hydrolysis. The reaction was initiated by the addition of substrate and subsequent incubation of the tubes in a shaking water bath for 10 min at 37°C . The reaction was terminated by the addition of 3 ml of chloroform/methanol (2:1, v/v) and 1 ml of 0.9% KCl. The tubes were mixed by vortexing and centrifuged to aid phase separation. The upper layer was removed and aliquots of the lower layer were evaporated under N_2 and redissolved in a smaller volume of chloroform/methanol (2:1, v/v). The lower layer was then applied to heat-activated TLC plates (110°C for 1 h) and the TLC plates were developed in chloroform/methanol/water/acetic acid (100:75:4:7, by vol.) [9]. The phospholipid bands were visualized with I_2 vapour and the band corresponding to lyso-PE was scraped into scintillation vials. The radioactivity associated with these bands was determined by scintillation counting in a Beckman LS3801 scintillation counter with Ecolite as the scintillant.

RESULTS AND DISCUSSION

PE-hydrolysing phospholipase A_1 activities have previously been reported in mammalian heart microsomes from hamster, dog and rat [6,10,11]. The presence of a similar enzyme activity in guinea-pig heart microsomes was established by incubating 1-palmitoyl-2-[^{14}C]linoleoyl-GPE with microsomes and monitoring the production of radiolabelled lyso-PE. To confirm that the hydrolysis was due to phospholipase A_1 activity, the substrate was incubated with 50 units of phospholipase A_2 and the production of radiolabelled lyso-PE was monitored [12]. After a 30 min incubation no labelling was detected in the lyso-PE fraction, an indication that the purity of the PE substrate was greater than 99% as specified by the manufacturer. The lyso-PE formed after incubating 1-palmitoyl-2-linoleoyl-GPE with microsomes was characterized by extracting it and subjecting it to hydrolysis by guinea-pig pancreatic phospholipase A_1 as previously described [12]. Parallel assays were conducted with 1-[^{14}C]palmitoyl-GPC. No release of labelled fatty acid was detected in assays with the lyso-PE fractions, whereas labelled fatty acid was released from the 1-palmitoyl-GPC substrate. Thus the lyso-PE formed was the 2-acyl-GPE species that is

formed by phospholipase A_1 activity and not 1-acyl-GPE formed by phospholipase A_2 hydrolysis of a 1-acyl-labelled contaminant of the substrate.

The pH profile for the hydrolysis of PE (Figure 1) conducted in the absence of detergent revealed a pH optimum of 9.0. The time course of the reaction and the activity as a function of substrate concentration are also shown in Figure 1. The reaction was linear for up to 15 min and maximum activity was obtained at concentrations of $200\text{--}300\text{ }\mu\text{M}$. The effects of detergents (sodium deoxycholate, sodium taurodeoxycholate, Triton X-100 and Triton QS-15) on the hydrolytic activity were examined. While Triton X-100 and Triton QS15 inhibited the hydrolysis, taurodeoxycholate and deoxycholate did not activate or inhibit the activity. In order to eliminate any differences in the solubility of the different PE substrates used in the study, all further experiments were conducted with mixed micelles of PE and taurodeoxycholate at a final detergent concentration of 1 mM . Under identical conditions, 1-stearoyl-2-arachidonoyl-GPE was hydrolysed at a rate of 56 nmol/h per mg of protein, 1-palmitoyl-2-arachidonoyl-GPE was hydrolysed at 34.5 nmol/h per mg of protein, 1-palmitoyl-2-oleoyl-GPE at 26 nmol/h per mg of protein and 1-palmitoyl-2-linoleoyl-GPE at 24 nmol/h per mg of protein.

Isoprenaline-mediated stimulation of phospholipase A activity in canine cardiac microsomes has been previously reported [6], although it was not established whether the phospholipase A activity was of the A_1 or A_2 type. We therefore investigated whether isoprenaline had any effect on guinea-pig heart microsomal phospholipase A_1 . As shown in Table 1, isoprenaline (1 pM – 1 mM) stimulated phospholipase A_1 hydrolysis of 1-palmitoyl-2-linoleoyl-GPE by up to 46%. To eliminate the possibility that these observations were due to the hydrolysis of PE by diacylglycerol or triacylglycerol lipase, the activities of these enzymes were determined under identical conditions to those used to assay phospholipase A_1 but with radiolabelled diacylglycerol or triacylglycerol as substrates. Diacylglycerol lipase activity was not detected under the experimental conditions and while triacylglycerol lipase activity was measurable, it was not affected by preincubation with isoprenaline (1 nM – $1\text{ }\mu\text{M}$).

To define the types of adrenergic receptors mediating the stimulation, the effects of specific agonists and antagonists were investigated. Atenolol ($1\text{ }\mu\text{M}$), a β_1 -specific adrenergic antagonist [13,14], inhibited the phospholipase A_1 hydrolysis of 1-palmitoyl-2-linoleoyl-GPE by approx. 50%; however, when atenolol was combined with isoprenaline, the hydrolysis of the substrate by phospholipase A_1 was 40% greater than in controls with atenolol alone. Butoxamine ($1\text{ }\mu\text{M}$), a β_2 -adrenergic-receptor-specific antagonist [13,14], did not affect phospholipase A_1 activity but blocked the isoprenaline-mediated stimulation. These results indicate that the hydrolysis of 1-palmitoyl-2-linoleoyl-GPE by phospholipase A_1 is specifically linked to β_2 -adrenergic receptor activation. The specificity was further confirmed in studies which showed that neither the α_1 -agonist phenylephrine [13] nor the α_2 -agonist clonidine [13] had any effect on the phospholipase A_1 hydrolysis of 1-palmitoyl-2-linoleoyl-GPE. The hydrolysis of 1-stearoyl-2-arachidonoyl and 1-palmitoyl-2-arachidonoyl-GPE was unaffected by the presence of isoprenaline.

In the previous study on isoprenaline-mediated stimulation of phospholipase A activity in canine cardiac microsomes, the mechanism responsible for the enhancement of phospholipase A activity was not investigated, but it was suggested that modulation of the phospholipase A activity occurred via a cascade comprising β -adrenergic receptors, cyclic AMP and phosphorylation [6]. In the light of the fact that activated cyclic AMP-dependent protein kinases are not found in microsomes

Figure 1 Characterization of phospholipase A₁ activity in guinea-pig heart microsomes

(a) Effect of pH. Phospholipase A₁ activity in guinea-pig heart microsomes was assayed as a function of pH. The reaction mixture contained 100 mM buffer (Tris/succinate, pH 6; Tris/HCl, pH 7–10), 100 μM 1-palmitoyl-2-linoleoyl-GPE prepared in the absence of detergent and 100 μg of heart microsomes in a total volume of 500 μl. The assay was performed as described in the Experimental section. The values are the means of two experiments each performed with three different preparations. (b) Time course of phospholipase A₁ activity. The assay mixture consisted of 300 μM 1-palmitoyl-2-linoleoyl-GPE prepared with taurodeoxycholate, 100 μg of protein and 100 mM Tris/HCl, pH 9.0. (c) Effect of PE concentration on phospholipase A₁ activity. Reaction mixtures were as described for (b) with different concentrations of 1-palmitoyl-2-linoleoyl-GPE. Reaction time was 10 min.

Table 1 Effect of adrenergic agonists and antagonist on guinea-pig heart microsomal phospholipase A₁ activity

Phospholipase A₁ activity was assayed in the presence of the indicated additives. The assays were performed as indicated in the Experimental section with the exception that guinea-pig heart microsomes were preincubated for 10 min at room temperature with the additives prior to initiation of the assay by the addition of substrate. The values are presented as percentages of the activity with no additives and represent the means ± S.D. of two experiments each done in triplicate. The specific activity of phospholipase A₁ for the hydrolysis of 1-palmitoyl-2-linoleoyl-GPE with no additive was 21 ± 3 nmol of lyso-PE produced/h per mg of protein.

Addition	Activity (% of control)
None	100
1 mM Isoprenaline	139 ± 7
1 μM Isoprenaline	140 ± 13
1 nM Isoprenaline	146 ± 8
1 pM Isoprenaline	127 ± 12
1 fM Isoprenaline	89 ± 14
1 μM Propranolol	104 ± 10
1 nM Isoprenaline + 1 μM propranolol	90 ± 15
100 μM Phenylephrine	98 ± 10
100 μM Clonidine	108 ± 14

Table 2 Effect of nucleotides on phospholipase A₁ activation

Phospholipase A₁ activity was assayed in the presence of the indicated additives using 1-palmitoyl-2-linoleoyl-GPE as the substrate. The additives were incubated with the microsomes for 10 min at room temperature prior to the initiation of the reaction by the addition of substrate. The values represent the means ± S.D. of three experiments each performed in triplicate. The specific activity of the phospholipase A₁ activity in the absence of any additive was 20 ± 3 nmol of lyso-PE produced/h per mg of protein.

Addition	Activity (% of control)
None	100
10 nM GTP[S]	111 ± 17
1 μM GTP[S]	169 ± 15
100 μM GTP[S]	142 ± 15
1 μM p[NH]ppG	143 ± 9
1 μM GDP[S]	92 ± 7
1 μM ATP[S]	95 ± 8
1 mM ATP[S]	85 ± 7
1 fM Isoprenaline	83 ± 10
1 nM Isoprenaline	152 ± 22
10 nM GTP[S] + 1 fM isoprenaline	173 ± 19
1 μM GTP[S] + 1 nM isoprenaline	141 ± 12
1 μM GDP[S] + 1 nM isoprenaline	87 ± 9

[15,16], another mechanism seemed more appropriate. One possibility was that the stimulation of phospholipase A₁ activity in response to adrenergic stimulation could occur via a G-protein, since β-adrenergic receptors are coupled to G-proteins [17] and many phospholipases are modulated by G-proteins [18]. To investigate this possibility we examined the effects of nucleotides and isoprenaline on the phospholipase A₁ activity (Table 2). The hydrolysis of 1-palmitoyl-2-linoleoyl-GPE by phospholipase A₁ was enhanced at guanosine 5'-[γ-thio]triphosphate (GTP[S]) concentrations between 1 and 100 μM. A similar level of activation was obtained with another non-hydrolysable analogue,

guanosine 5'-[β,γ-imido]triphosphate (p[NH]ppG) (1 μM), but no activation was obtained with guanosine 5'-[β-thio]diphosphate (GDP[S]), ATP or adenosine 5'-[γ-thio]triphosphate (ATP[S]) (1 μM–1 mM). No additive effects on the stimulation of phospholipase A₁ activity were observed when stimulatory concentrations of GTP[S] (1 μM) and isoprenaline (1 nM) were combined; however, the combined effect of GTP[S] (10 nM) and isoprenaline (1 fM) at concentrations that did not by themselves stimulate the hydrolysis of 1-palmitoyl-2-linoleoyl-GPE resulted in a 73% increase in the hydrolysis of the substrate by

phospholipase A_1 (Table 2). In contrast, the addition of GDP[S] ($1 \mu\text{M}$), an established inhibitor of G-protein activation [19], blocked the isoprenaline-mediated stimulation of the hydrolysis of 1-palmitoyl-2-linoleoyl-GPE by phospholipase A_1 (Table 2).

Taken together, the above results provide indirect evidence that the isoprenaline-mediated stimulation of the hydrolysis of 1-palmitoyl-2-linoleoyl-GPE by phospholipase A_1 is mediated by G-proteins. Unequivocal proof would require purification of the putative G-proteins, enzyme and receptors for reconstitution studies. In the light of evidence that $\beta\gamma$ subunits of G-proteins may, like the α subunit, modulate effector function, we cannot rule out a situation analogous to that in retinal rod membranes, where $G\alpha_i$ of transducin stimulates cyclic GMP phosphodiesterase while the $\beta\gamma$ subunits activate phospholipase A_2 [20]. Our studies do not allow any speculation on whether the putative G-protein interacts directly with phospholipase A_1 or acts via an intermediary protein. The hydrolysis of 1-stearoyl-2-arachidonoyl-GPE by phospholipase A_1 activity in the microsomes was not affected by the addition of GTP[S] or p[NH]ppG.

Observations that the hydrolysis of 1-stearoyl-2-arachidonoyl-GPE by phospholipase A_1 was unaffected by isoprenaline or guanine nucleotides, even though the rate of hydrolysis of this substrate was more than 2-fold greater than the rate of hydrolysis of 1-palmitoyl-2-linoleoyl-GPE, led to studies investigating the possibility that distinct activities may be responsible for the hydrolysis of the two substrates. Microsomal membranes in buffer with protease inhibitors (PMSF, leupeptin, aprotinin) were incubated at 60°C for selected periods (0–5 min) and cooled on ice for an additional 5 min before the addition of either 1-palmitoyl-2-linoleoyl-GPE or 1-stearoyl-2-arachidonoyl-GPE to assay for enzyme activity. Hydrolysis of 1-palmitoyl-2-linoleoyl-GPE was undetectable after 1 min of heat treatment, while the rate of hydrolysis of 1-stearoyl-2-arachidonoyl-GPE was decreased to 66% of control values after 1 min. Thus hydrolysis of 1-palmitoyl-2-linoleoyl-GPE was more heat-labile than that of 1-stearoyl-2-arachidonoyl-GPE.

To determine the effect of 1-stearoyl-2-arachidonoyl-GPE on the hydrolysis of 1-palmitoyl-2-linoleoyl-GPE, the rate of hydrolysis of 1-palmitoyl-2-linoleoyl-GPE in mixed micelles of 1-palmitoyl-2- ^{14}C linoleoyl-GPE ($150 \mu\text{M}$) and 1-stearoyl-2-arachidonoyl-GPE ($150 \mu\text{M}$) was compared with the hydrolysis of 1-palmitoyl-2-linoleoyl-GPE alone ($150 \mu\text{M}$). The results showed that when presented alone the rate of hydrolysis of 1-palmitoyl-2-linoleoyl-GPE was 22 ± 2 nmol of lyso-PE produced/h per mg of protein, compared with 22 ± 7 nmol of lyso-PE produced/h per mg of protein when presented as mixed micelles with 1-stearoyl-2-arachidonoyl-GPE. The inability of 1-stearoyl-2-arachidonoyl-GPE to inhibit the hydrolysis of 1-palmitoyl-2-linoleoyl-GPE, coupled with the differential heat sensitivities, suggests the possible existence of different isoforms of the enzyme that are differentially regulated. Hence the search for putative signal-activated phospholipase A_1 in cells may require strategies that discriminate between different molecular species of phospholipids. It is worth noting that a recently purified bovine brain cytosolic phospholipase A_1 was 12-fold more active with the same molecular species of PE relative to PC [21], an indication perhaps that this is a PE-specific phospholipase. We were also able to demonstrate that the presence of 150 or $180 \mu\text{M}$ 1-palmitoyl-2-linoleoyl-GPC did not inhibit the hydrolysis of equal or lower concentrations of 1-palmitoyl-2-linoleoyl-GPE (50 – $150 \mu\text{M}$). Indeed, stimulation of the hydrolysis of 1-palmitoyl-2-linoleoyl-GPE was observed in the presence of the PC species (Figure 2), indicating that there is no competition between the analogous PC and PE species for the active site on the PE-hydrolysing phospholipase A_1 .

Figure 2 Effect of 1-palmitoyl-2-linoleoyl-GPC on the hydrolysis of 1-palmitoyl-2-linoleoyl-GPE by guinea-pig heart microsomal phospholipase A_1

The rate of hydrolysis of 1-palmitoyl-2- ^{14}C linoleoyl-GPE (50 – $150 \mu\text{M}$) was measured in the presence of $0 \mu\text{M}$ ($\blacksquare$), $150 \mu\text{M}$ ($\bullet$) or $180 \mu\text{M}$ ($\blacktriangle$) 1-palmitoyl-2-linoleoyl-GPC. The PE and PC substrates were added separately to the reaction mixture. The assay was performed as described in the Experimental section, with the exception that the reaction was initiated by the addition of microsomal protein. The values represent the means of two independent experiments each performed in duplicate. The S.D. for each point was less than 15% of the mean. * $P < 0.05$ relative to the corresponding value with $0 \mu\text{M}$ 1-palmitoyl-2-linoleoyl-GPC, as determined by Student's t test.

Table 3 Effect of Ca^{2+} and EGTA on the isoprenaline- and GTP[S]-mediated activation of guinea-pig heart microsomal phospholipase A_1

Phospholipase A_1 activity was assayed in the presence of the additives indicated in combination or alone. The phospholipase A_1 assay was performed as described in the Experimental section with 1-palmitoyl-2-linoleoyl-GPE. The microsomes were preincubated with the additives for 10 min at room temperature prior to initiation of the assay. The values are presented as percentages of the phospholipase A_1 activity observed without any additives. The values represent the means $\pm$ S.D. of three experiments each done in triplicate. The specific activity of the phospholipase A_1 in the absence of any additives was 23 ± 3 nmol of lyso-PE formed/h per mg of protein. *Significantly different from controls with no addition by Student's t test at a level of $P < 0.005$.

Addition	Activity (% of control)
None	100
1 nM Isoprenaline	$157 \pm 13^*$
100 μM Ca^{2+}	$165 \pm 18^*$
2 mM EGTA	$83 \pm 10^*$
100 μM Ca^{2+} + 1 nM isoprenaline	$140 \pm 9^*$
2 mM EGTA + 1 nM isoprenaline	93 ± 10
1 μM GTP[S]	$148 \pm 15^*$
100 μM Ca^{2+} + 1 μM GTP[S]	$144 \pm 11^*$
2 mM EGTA + 1 μM GTP[S]	102 ± 9

Hamster [8] and rat heart [6] phospholipase A_1 activities are activated by high concentrations of Ca^{2+} (5 mM) while canine heart phospholipase A_1 activity is inhibited by Ca^{2+} and activated by EDTA [6]. In the present study we observed a slight decrease (15%) in the hydrolysis of both 1-palmitoyl-2-linoleoyl-GPE and 1-stearoyl-2-arachidonoyl-GPE by phospholipase A_1 in incubations with EGTA (2 mM), indicating that the activity was not obligatorily dependent on the availability of Ca^{2+} (Table 3).

However, the addition of 100 μ M Ca²⁺ resulted in a 65% and 50% increase in the hydrolysis of 1-palmitoyl-2-linoleoyl-GPE and 1-stearoyl-2-arachidonoyl-GPE respectively relative to controls. The level of enhancement of 1-palmitoyl-2-linoleoyl-GPE hydrolysis by Ca²⁺ was similar to that produced by isoprenaline. The combination of isoprenaline and Ca²⁺ did not result in an additive or synergistic effect. EGTA (2 mM) completely abolished the isoprenaline-mediated stimulation of phospholipase A₁ hydrolysis of 1-palmitoyl-2-linoleoyl-GPE. The activation of the phospholipase A₁ by GTP[S] (1 μ M) was also not observed in the presence of 2 mM EGTA (Table 3). Furthermore the combination of Ca²⁺ and GTP[S] did not stimulate the hydrolysis of 1-palmitoyl-2-linoleoyl-GPE beyond that obtained with the individual additives. The above results suggest that even though the hydrolysis of PE by phospholipase A₁ is not Ca²⁺-dependent, both the isoprenaline- and guanine nucleotide-mediated enhancements of phospholipase A₁ hydrolysis of 1-palmitoyl-2-linoleoyl-GPE appear to require cations since the stimulation was abolished by EGTA. The levels of cations required appear to be very low, as quantities present in the microsomal fractions prepared from tissue homogenized in buffers containing 1 mM EDTA were sufficient for the GTP[S]- and isoprenaline-mediated stimulatory effects. Since stimulation of phospholipase A₁ activity appears to be mediated by a G-protein, the inhibition of isoprenaline stimulation by EGTA probably reflects a cation requirement at the level of G-protein-mediated activation. A Ca²⁺ requirement for stimulation of phospholipase A₂ activity by GTP analogues has been observed in neutrophils [22].

The selective release of fatty acids from phospholipids is a key step in the regulation of the synthesis of cellular mediators [23]. Studies have implicated cytosolic phospholipase A₂ as a key molecule in arachidonate release from phospholipids in cells [24,25]. In the guinea-pig heart this enzyme was not detected by Western blot analysis [25], suggesting that alternative pathways mediate the release of fatty acids in this tissue. One possible pathway may involve the sequential action of phospholipase A₁ and lysophospholipase A₂ [12,26,27]. We have previously demonstrated the presence of lysophospholipases that display selectivity towards different species of 1-lyso-2-acyl-GPE in guinea-pig heart microsomes [27]. The results obtained in the present study suggest that the guinea-pig heart contains multiple phospholipases A₁ that could be differentially regulated to hydrolyse particular molecular species of PE; these in turn could be hydrolysed by the lysophospholipases to release specific fatty acids such as arachidonic and linoleic acids. Linoleic acid has

been postulated to activate protein kinase C [4,28,29] and may also be converted into lipoxygenase metabolites [30].

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