Biochem. J. (1992) 284, 917–922 (Printed in Great Britain)

G-protein-mediated activation of turkey erythrocyte phospholipase C by β-adrenergic and P_{2y}-purinergic receptors

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Isoprenaline, previously known only to stimulate adenylate cyclase via the stimulatory G-protein, G_s, activates turkey erythrocyte ghost phospholipase C (PLC) in a dose-dependent manner when GTP or guanosine 5'-[γ-thio]triphosphate (GTP[S]) is present. The effect is specific in that it is abolished by β-adrenergic-receptor antagonists. Stimulation of adenosine receptors, which also couple to adenylate cyclase via G_s in turkey erythrocytes, does not activate PLC, indicating that the stimulation observed in the presence of isoprenaline is not due to G_s activation. Furthermore, the stimulation seen is independent of cyclic AMP production. Purified turkey erythrocyte PLC is activated in an adenosine 5'-[β-thio]diphosphate (ADP[S]; a P_{2y}-purinergic-receptor agonist)- or isoprenaline-regulated manner when reconstituted with turkey erythrocyte ghosts, demonstrating that a single species of PLC effector enzyme can be regulated by both the purinergic and the β-adrenergic receptor populations present in turkey erythrocyte membranes. Pretreatment of intact turkey erythrocytes with the P_{2y} agonist ADP[S] causes decreased PLC responsiveness of subsequent ghost preparations to ADP[S] stimulation, although responses to isoprenaline are unaffected (homologous desensitization). In contrast, pretreatment of intact erythrocytes with isoprenaline results in heterologous desensitization of both the P_{2y} and the β-adrenergic receptors. These effects occur at the level of receptor–G-protein coupling, since PLC stimulation by GTP[S] (which directly activates G-proteins) in the absence of agonists is unaffected.

INTRODUCTION

The regulation of phospholipase C (PLC) by guanine nucleotides and P_{2y}-purinergic-receptor agonists has been extensively characterized in ghosts prepared by hypo-osmotic lysis of [^{3}H]inositol-labelled turkey erythrocytes [1]. Receptor-stimulated activation of turkey erythrocyte PLC (and of PLCβ isoenzymes in other cells) is thought to be mediated by a heterotrimeric G-protein [2–4]. The agonist-occupied receptor is believed to catalyse the exchange of GDP bound to the α subunit of the G-protein (Gα) for free GTP, which possibly results in dissociation of the α from the βγ subunits. The GTP-liganded α subunit is then thought to regulate effector-enzyme (PLC) activity. Hydrolysis of the GTP by an endogenous GTPase activity returns Gα to its inactive GDP-liganded state [5].

Boyer *et al.* [6] investigated the effects of exogenously added G-protein βγ subunits on purinergic-receptor-regulated PLC activity in turkey erythrocyte ghosts. It was found that, when reconstituted with turkey erythrocyte ghost 'acceptor' membranes, βγ subunits potentiated P_{2y}-purinergic-agonist- and GTP-stimulated PLC activity. To explain this result, the authors proposed that successful coupling with the P_{2y} receptor required the G-protein that stimulates PLC (G_{PLC}) to exist in its undissociated heterotrimeric (αβγ) form. They suggested that the reconstituted βγ subunits might be interacting with free α subunits of G_{PLC} (α_{PLC}) in the membranes, and by mass action driving the formation of more G-protein heterotrimers available for coupling with the agonist-occupied P_{2y} receptor. Selective dissociation, by agonist and GTP, of heterotrimeric G-proteins which couple to other receptors in erythrocyte ghosts may result in an increased free βγ-subunit content of the plasma membranes, and might similarly increase the ability of the P_{2y} receptor to stimulate PLC. We therefore investigated potential interactions between β-adrenergic receptors [known to activate adenylate cyclase via the stimulatory G-protein (G_s) in turkey erythrocyte membranes] and P_{2y} receptors, with respect to G-protein-mediated PLC activation. Surprisingly, the results indicate that turkey erythrocyte β-adrenergic receptors can activate PLC via a G_s-independent mechanism. The results are consistent with direct stimulation of PLC by a G_{PLC}-coupled β-adrenergic receptor.

MATERIALS AND METHODS

Materials

Isoprenaline and propranolol were from Sigma. Other β-receptor antagonists were kindly given by Dr. A. L. Willcocks and Dr. T. K. Harden. Purified turkey erythrocyte cytosolic PLC was generously provided by Dr. A. J. Morris, Mr. G. Waldo and Dr. T. K. Harden. All other materials were from previously specified sources [1].

Erythrocytes

Turkey erythrocytes were collected, washed and lysed as previously described [1].

Labelling of erythrocyte ghost phosphoinositides with [^{3}H]inositol

We routinely labelled 150 μl of packed washed erythrocyte ghosts with 100–200 μCi of [^{3}H]inositol for 1 h at 37 °C, in a final volume of 200 μl containing 20 mM-Hepes (pH 7.8), 10 mM-$MgCl_2$ and 1 mM-CMP. This procedure generally incorporates 35–45% of the labelled inositol into ghost membrane PtdIns by a combination of PtdIns synthase and PtdIns/*myo*-inositol headgroup exchange reactions [7]. MgATP (10 μl of 200 mM) was then added to the reaction mixture, which was incubated for a further 12 min. This results in the equilibration of the labelled PtdIns pool with PtdIns*P* and PtdInsP_2 owing to the concerted actions of the ghost-associated phosphoinositide kinases and phosphatases [1]. The labelled ghosts were then washed three

Abbreviations used: G_s, stimulating G-protein; PLC, phospholipase C; ADP[S], adenosine 5'-[β-thio]diphosphate; GTP[S], guanosine 5'-[γ-thio]triphosphate; Ins*P*s, inositol phosphates; G_{PLC}, G-protein that stimulates PLC.

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times in 40 ml of ice-cold lysis buffer [1] by centrifugation at 13500 rev./min (Beckman JA-20 rotor) for 1 min. After a final wash with 40 ml of ice-cold 10 mM-Hepes (pH 7.0)/5 mM-$MgCl_2$, the ghosts were resuspended and diluted in this buffer. The ghosts were stored on ice for up to 45 min before assay of PLC activity.

PLC assay

PLC activity was assayed in an intracellular-type buffer [1] in a final volume of 200 μl containing the indicated concentrations of drugs. Ghosts and assay buffer were warmed for 2 min immediately before assays. Assays were terminated, and [^{3}H]Ins*P*s and phosphoinositides were extracted and quantified as described previously [1].

Reconstitution of purified turkey erythrocyte cytosolic PLC with [^{3}H]inositol-labelled ghost membranes

Washed intact turkey erythrocytes were labelled for 18–20 h with [^{3}H]inositol, and acceptor membranes were prepared exactly as previously described [8]. In brief, the labelled cells were lysed and washed extensively with lysis buffer. The washed ghost pellet was then homogenized in 40 vol. of Mg^{2+}-free 10 mM-Hepes (pH 7.0). The ghosts were pelleted (JA-20 rotor, 13500 rev./min, 1 min) and diluted to the indicated protein concentration in this buffer. The ghosts were then mixed with the indicated amounts of purified PLC [9], or 10 mM-Hepes for control, and added to pre-warmed tubes containing assay buffer and drugs, to give a final volume of 200 μl. Production of [^{3}H]Ins*P*s was measured as described above.

Desensitization of purinergic and β-adrenergic receptors

Intact erythrocytes were labelled with [^{3}H]inositol as described above. The labelled cells were washed in Dulbecco's modified Eagle's medium (DMEM) by repeated centrifugation at 1000 *g* for 5 min. The washed erythrocytes were diluted in DMEM to give a 20% (approx.) cell suspension. A 400 μl portion of the labelled cell suspension was incubated with or without agonists at 37 °C for the indicated times. The cells were then lysed by rapid dilution and mixing in 40 ml of ice-cold lysis buffer. Ghosts were pelleted by centrifugation at 13500 rev./min (JA-20 rotor) for 1 min, and subjected to two further washes in 40 ml of lysis buffer, followed by a final wash in 40 ml of 10 mM-Hepes (pH 7.0)/5 mM-$MgCl_2$. The ghost pellets were then resuspended and normalized to the same volumes in this buffer. PLC assays were carried out on these preparations as described above.

The results are presented as means of duplicates, and are representative of experiments which were repeated at least twice, as indicated by the Figure legends.

RESULTS

We investigated the effects of the β-adrenergic agonist isoprenaline on PLC activity stimulated by adenosine 5′-[β-thio]diphosphate (ADP[S]) (a P_{2y}-purinergic-receptor agonist) and GTP in [^{3}H]inositol-labelled turkey erythrocyte ghosts. It was found that isoprenaline increased the Ins*P* response produced by ADP[S] and GTP (Table 1); however, the enhanced PLC activity seen in the presence of isoprenaline was also evident in the absence of ADP[S], indicating that isoprenaline was not promoting coupling between the P_{2y} receptor and G_{PLC}. Stimulation of adenosine receptors, which activate adenylate cyclase via G_s in turkey erythrocytes [10], had no effect on GTP- or GTP+ADP[S]-stimulated PLC activity, indicating that G_s activation was not responsible for the enhanced PLC activity seen in the presence of isoprenaline. The turkey erythrocyte ghosts are depleted of cytosolic components (e.g. ATP and cyclic AMP-dependent protein kinase), indicating that the stimulation of PLC is independent of adenylate cyclase activation. Furthermore, inclusion of 1–5 mM-cyclic AMP or -dibutyryl cyclic AMP in the assays had no effect on PLC activity (results not shown).

The stimulatory effect of isoprenaline on PLC activity exhibited

Table 1. Enhancement of guanine-nucleotide-stimulated PLC activity by isoprenaline and ADP[S]

Turkey erythrocyte ghosts were labelled with [^{3}H]inositol as described in the Materials and methods section. PLC activity was assayed by measuring [^{3}H]Ins*P* formation after 4 min at 37 °C after stimulation of the labelled ghosts with agonists and guanine nucleotides at the following concentrations: GTP, 1 mM; ADP[S], 100 μM; isoprenaline, 100 μM; adenosine, 100 μM; GTP[S], 10 μM. Each assay contained approx. 1×10^6 d.p.m. of total ^{3}H-labelled ghost phosphoinositides. The data show actual values obtained in a representative experiment, and represent d.p.m. of Ins*P*s obtained in duplicate assays. To provide an indication of inter-experimental variation, the values in parentheses represent pooled data from three separate membrane preparations; PLC activity is expressed as percentage of activity (±S.E.M.) produced by a combined dose of GTP[S], isoprenaline and ADP[S].

Stimulus	[^{3}H]Ins*P* production (d.p.m.)	
Basal	4202±198	(2.7±1.30)
Isoprenaline	4457±327	(2.8±0.88)
GTP	13280±439	(9.34±2.02)
GTP+ADP[S]	20357±24	(15.92±0.06)
GTP+ADP[S]+isoprenaline	26221±147	(19.56±0.59)
GTP+isoprenaline	18182±496	(13.61±0.70)
GTP+ADP[S]+adenosine	19360±1120	(14.50±0.50)
GTP+adenosine	13244±373	(9.20±1.01)
GTP[S]	62450±371	(42.70±4.00)
GTP[S]+ADP[S]	111614±2476	(83.8±0.57)
GTP[S]+ADP[S]+isoproterenol	128474±1624	(100)
GTP[S]+isoproterenol	89175±590	(65.04±5.27)
GTP[S]+ADP[S]+adenosine	110148±675	(84.9±390)
GTP[S]+adenosine	62692±304	(44.86±2.60)

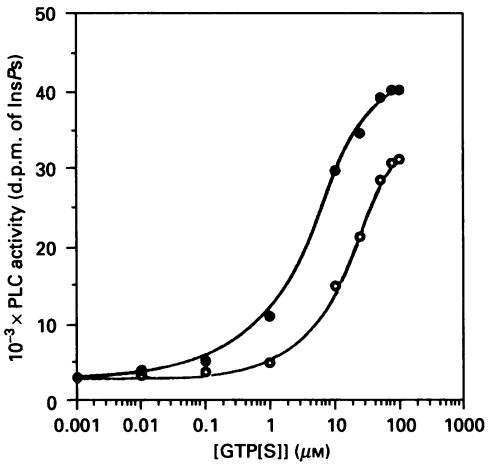

Fig. 1. Dose–response relationship for GTP[S]-stimulated PLC activity in the absence and presence of a maximally effective dose of isoprenaline

[^{3}H]Inositol-labelled erythrocyte ghosts were assayed for GTP[S]-stimulated PLC activity at the indicated concentrations of the guanine nucleotide, in the absence (○) or presence (●) of 100 μM-isoprenaline. Assays were carried out at 37 °C for 4 min. Each data point is the mean of duplicate incubations which differed by less than 5%. The data shown are representative of results obtained in three separate experiments.

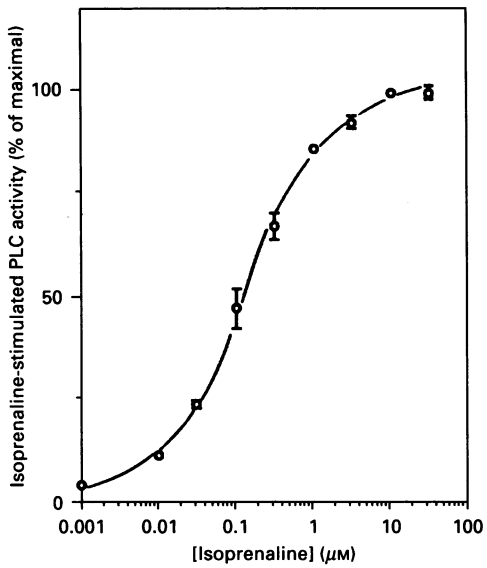

Fig. 2. Dose–response relationship for isoprenaline-stimulated PLC activity

[^{3}H]Inositol-labelled turkey erythrocyte ghosts were assayed for PLC activity in the presence of 10 μM-GTP[S] and the indicated concentrations of isoprenaline. Assays were terminated after 4 min at 37 °C. The data are expressed as percentages of maximum isoprenaline-stimulated PLC activity, and are representative of three separate experiments. Each data point is the mean of duplicate incubations.

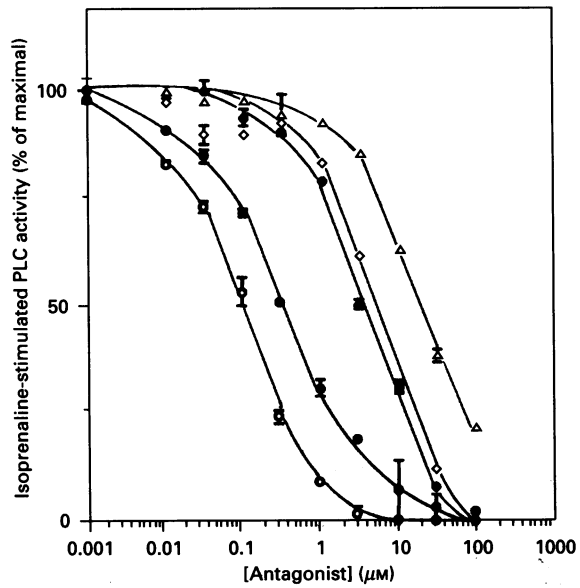

Fig. 3. Inhibition of isoprenaline-stimulated PLC activity by various β-adrenergic-receptor antagonists

[^{3}H]Inositol-labelled ghosts were assayed for PLC activity for 4 min at 37 °C in the presence of 10 μM-GTP[S], 10 μM-isoprenaline, and the indicated concentrations of the following antagonists: propranalol (○), timolol (●), CGP 20172A (◇), ICI 118.551 (◆), betaxalol (△). Data are expressed as percentages of the PLC activity caused by isoprenaline in the absence of any antagonist. Each data point is the mean of a duplicate incubation, and is from a single experiment which has been repeated twice with similar results.

an absolute requirement for guanine nucleotides {GTP or guanosine 5′-[γ-thio]triphosphate (GTP[S]); Table 1, Fig. 1}. Isoprenaline increased the maximal responsiveness of PLC activity caused by GTP[S], and decreased the EC_{50} value for stimulation of PLC activity by GTP[S] alone (Fig. 1). Isoprenaline increased GTP[S]-stimulated PLC activity in a dose-dependent manner (Fig. 2), with an EC_{50} of 126±16 nM ($n = 3$). The dose–response relationship for isoprenaline-stimulated PLC activity in the presence of 0.1 mM-GTP (results not shown) was indistinguishable (within the limits of experimental error) from that obtained in the presence of 10 μM-GTP[S]. However, since the non-hydrolysable guanine nucleotide elicited larger PLC responses, this was used in all subsequent experiments. AlF_4^--stimulated PLC was unaltered by the presence of 100 μM-ADP[S] or -isoprenaline (results not shown).

The isoprenaline-stimulated PLC activity was inhibited by a range of β-adrenergic-receptor antagonists. Of the antagonists tested, propranolol (a non-selective β-receptor antagonist) was the most potent at inhibiting isoprenaline-stimulated PLC activity (Fig. 3), with a K_i of 1.12±0.18 nM ($n = 3$). This agrees well with the reported K_d of 1.3 nM for propranolol displacement of [^{125}I]iodohydroxybenzylpindolol binding to turkey erythrocyte membranes [11]. The β2-selective antagonist ICI 118.551 and the β1-selective antagonists Betaxalol and CGP 20172A were able to inhibit PLC activity with K_i values of 37±6 nM, 139±31 nM and 74±12 nM respectively. Timolol, a hydrophilic non-selective β-receptor antagonist, potently inhibited the isoprenaline- and GTP[S]-stimulated PLC activity (with a K_i of 2.6±0.4 nM), thus indicating that the inhibition of β-receptor-stimulated PLC activity by the antagonists was unlikely to be a consequence of membrane-perturbing effects owing to the hydrophobic nature of these compounds. In support of this, GTP[S]- or ADP[S]+GTP[S]-stimulated PLC activity was not inhibited by maximally effective doses of any of the β-receptor antagonists (results not shown). Although the turkey erythrocyte β-receptor is described as being β1-like, it exhibits pharmacological properties distinct from those of mammalian β1 and β2 receptors [11]. It is not known whether a single β-receptor subtype is capable of stimulating both PLC and adenylate cyclase in turkey erythrocyte membranes. However, preliminary experiments indicate that a range of selective and non-selective β-adrenergic-receptor antagonists inhibit both isoprenaline-stimulated PLC and adenylate cyclase with the same rank order of potencies (C. Vaziri & T. K. Harden, unpublished work).

Activation of G-proteins by non-hydrolysable GTP analogues is preceded by a noticeable time lag [12], presumed to be due to the slow dissociation of GDP from the G-protein. Agonist-occupied receptors enhance the rate of exchange of G-protein-bound GDP for free guanine nucleotide [5], thereby decreasing the lag phase which precedes G-protein activation by non-hydrolysable analogues of GTP. As shown in Fig. 4, ADP[S] and isoprenaline both decreased the lag phase of PLC activation observed with GTP[S] alone. ADP[S] was more effective than isoprenaline at both decreasing the lag phase and increasing the maximal attained rate of PLC activity. The effect was partially additive, in that combined maximally effective doses of ADP[S] and isoprenaline resulted in a decrease lag phase and a greater attained rate of PLC activity than that observed in the presence of either agonist alone. We have consistently observed incomplete additivity of the PLC response to combined maximally effective doses of isoprenaline and ADP[S]. In four separate experiments the Ins*P* responses to 100 μM-ADP[S] in the presence of 100 μM-isoprenaline were 87% (Table 1), 74% (Fig. 4, 4 min time point), 80% and 71% (results not shown) of a calculated fully additive response (the sum of individual ADP[S]- and isoprenaline-stimulated components of the PLC response in the absence of the other agonist). However, full additivity occurred at 0.1 μM-ADP[S] and 0.1 μM-isoprenaline in the presence of 10 μM-GTP[S] (Table 2).

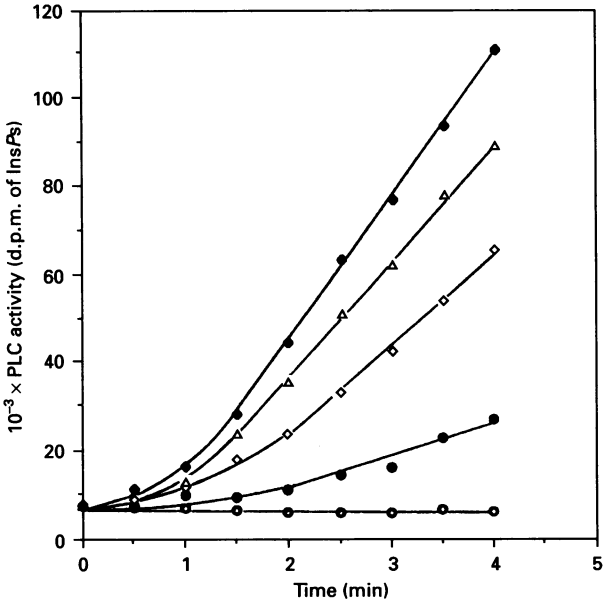

Fig. 4. Time course of activation of turkey erythrocyte ghost PLC by agonists and guanine nucleotides

[^{3}H]Inositol-labelled ghosts were assayed for PLC activity at 30 °C for the indicated times in the absence (○) or presence of the following agonists and guanine nucleotides at the indicated concentrations: 10 μM-GTP[S] (●); 10 μM-GTP[S] + 100 μM-ADP[S] (△); 10 μM-GTP[S] + 100 μM-isoprenaline (◇); 10 μM-GTP[S] + 100 μM-ADP[S] + 100 μM-isoprenaline (◆). Each data point is the mean of duplicate incubations which differed by less than 5%. The Figure shows a single experiment which was repeated twice with similar results.

Table 2. Additivity of P_{2y}-purinergic- and β-adrenergic-receptor-stimulated PLC activity at low concentrations of agonists

[^{3}H]Inositol-labelled ghosts were assayed for PLC activity for 4 min at 37 °C with agonists and guanine nucleotides at the following concentrations: GTP[S], 10 μM; ADP[S], 0.1 μM; isoprenaline, 0.1 μM. The data show actual values obtained in a representative experiment performed in duplicate. The values in parentheses express PLC activity as percentage of activity (±S.E.M.) produced by a combined dose of GTP[S], isoprenaline and ADP[S] (n = 3).

Stimulus	[^{3}H]Ins*P* production (d.p.m.)
Basal	7559±429 (5.91±1.30)
GTP[S]	32949±1140 (37.73±4.90)
GTP[S] + isoprenaline	54316±691 (57.94±4.0)
GTP[S] + ADP[S]	69436±1283 (73.36±1.78)
GTP[S] + isoprenaline + ADP[S]	86654±297 (100)

It was decided to determine whether stimulation of the P_{2y} and β-receptors activated the same molecular species of PLC enzyme. To address this, we examined the ability of purified turkey erythrocyte cytosolic PLC to interact in an isoprenaline- and GTP[S]-regulated manner with turkey erythrocyte ghosts. Morris *et al.* [9] purified a 150 kDa PLC from turkey erythrocyte cytosol. This enzyme was shown to reconstitute in an ADP[S]/guanine-nucleotide regulated manner with [^{3}H]inositol-labelled turkey erythrocyte ghosts which had undergone prior exposure to Mg^{2+}-free Hepes buffer [8]. Exposure to Mg^{2+}-free buffers uncouples the endogenous PLC activity from the G-protein, resulting in ghosts with markedly diminished ability to respond to agonists and guanine nucleotides (C. Vaziri, unpublished work). The 'uncoupled' ghosts, however, are competent to serve as acceptor

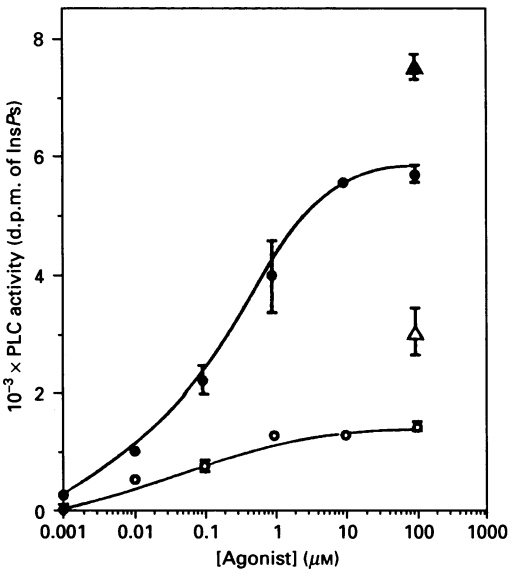

Fig. 5. Concentration-dependence of isoprenaline for stimulation of purified cytosolic PLC when reconstituted with turkey erythrocyte ghosts

'Uncoupled' acceptor ghosts (prepared as described in the Materials and methods section) were combined with purified turkey erythrocyte cytosolic PLC or with 10 mM-Hepes (pH 7.0). Samples (20 μl) containing 15 μg of ghost protein (approx. 100000 d.p.m. of [^{3}H]phosphoinositides) without (○, △) or (●, ▲) 150 ng of PLC, were then added to prewarmed assay buffer to give a final assay volume of 200 μl. Assays contained 10 μM-GTP[S] and the indicated concentrations of isoprenaline (○, ●) or ADP[S] (△, ▲). Assays were terminated after 5 min at 37 °C. The Ins*P* accumulation caused by GTP[S] alone has been subtracted from the data shown. This result was also observed in a separate experiment.

membranes for exogenously added PLC, which is activated in an agonist/guanine-nucleotide-regulated manner ([8]; C. Vaziri, unpublished work). Fig. 5 shows that purified cytosolic PLC is activated by isoprenaline in a dose-dependent manner when reconstituted with 'uncoupled' ghosts in the presence of 10 μM-GTP[S]. The isoprenaline dose-dependency of reconstituted PLC activity is identical with that of the endogenous PLC in fully coupled ghosts. These results demonstrate that a single species of PLC effector enzyme can be regulated by stimulation of both the P_{2y}-purinergic and the β-adrenergic receptors.

Martin & Harden [13] reported that exposure of intact turkey erythrocytes to purinergic agonists resulted in desensitization of ADP[S]-stimulated PLC responses in subsequently prepared ghost membranes. In agreement with these results, brief exposure of [^{3}H]inositol-labelled erythrocytes to 100 μM-ADP[S], followed by rapid lysis of the cells and extensive washing of the resultant ghosts, produced ghost membranes with diminished ability to generate [^{3}H]Ins*P*s in response to ADP[S] and GTP[S], the ADP[S]-stimulated component being decreased to 66% of that seen in control preparations (Table 3). However, the ability of GTP[S] or GTP[S] + isoprenaline to stimulate PLC activity was unaltered in these ghost preparations, indicating that G-protein/PLC coupling remained intact, and that homologous desensitization occurred at the level of the P_{2y}-receptor/G-protein interaction. In contrast, pre-exposure of cells to isoprenaline resulted in heterologous desensitization of the P_{2y}- and the β-adrenergic-stimulated PLC activities in subsequently prepared ghost membranes. GTP[S]-stimulated PLC activity was not significantly altered in ghosts prepared from isoprenaline-pretreated cells; however, ADP[S]- and isoprenaline-stimulated components of PLC activity were decreased to 58% and 34% of control values

Table 3. Desensitization of agonist-stimulated PLC activity in ghost membranes prepared from agonist-pretreated intact erythrocytes

Intact turkey erythrocytes were labelled overnight with [^{3}H]inositol as described in the Materials and methods section. The labelled erythrocytes were washed repeatedly in DMEM. Samples (400 μl) of labelled cell suspension were incubated at 37 °C for 20 min in the absence or presence of 100 μM-ADP[S] or 100 μM-isoprenaline. After cell lysis and extensive washing of the resultant ghosts, the preparations were assayed for PLC activity at 37 °C for 4 min. Agonist and guanine nucleotide concentrations were as follows: GTP[S], 10 μM; ADP[S], 100 μM; isoprenaline, 100 μM. Assays contained approx. 1.1×10^6 d.p.m. of total [^{3}H]phosphoinositides. The data shown are from a single experiment which was performed in duplicate. The values in parentheses express PLC activity (±S.E.M.) as a percentage of the GTP[S]-stimulated activity in ghost membranes prepared from cells preincubated in the absence of agonist, and represent data from three separate experiments.

	100 × d.p.m. in [^{3}H]Ins*P*s/d.p.m in [^{3}H]lipids		
	Control	ADP[S]-pretreated	Isoprenaline-pretreated
Basal	0.14±0.02 (0.09)	0.14±0.01 (0.10)	0.14 (0.10)
GTP[S]	1.60±0.01 (100)	1.69±0.03 (107.5±2.04)	1.50±0.01 (94.5±1.2)
GTP[S]+ADP[S]	7.00±0.04 (383.5±43.7)	5.26±0.06 (301.5±21.66)	4.62±0.00 (281.5±6.5)
GTP[S]+isoprenaline	5.22±0.04 (290.5±29.0)	5.15±0.06 (294.1±22.0)	2.73±0.01 (170±7.6)

respectively. The [^{3}H]lipid content of the ghost membranes and the distribution of label between PtdIns, PtdIns*P* and PtdInsP_2 were unaffected by pretreatment of the intact cells with agonists (ADP[S] or isoprenaline). This presumably reflects the rapid resynthesis of these lipids after agonist treatment of the intact cells, which contain all the enzymes necessary to recycle Ins*P*s to PtdIns and polyphosphoinositides [1].

DISCUSSION

We have investigated the regulation of G-protein-activated PLC by P_{2y}-purinergic- and β-adrenergic-receptor agonists in turkey erythrocyte ghosts. A significant methodological difference between the experiments reported here and previous studies of turkey erythrocyte PLC regulation [1,6,9,12,13,14] lies in the labelling protocol employed to incorporate [^{3}H]inositol into ghost membrane phosphoinositides. Whereas previous studies have been carried out on ghosts prepared by the hypo-osmotic lysis of [^{3}H]inositol-labelled intact erythrocytes, we have directly labelled erythrocyte ghost phosphoinositides to high specific radioactivity *in vitro*. By exploiting the PtdIns synthase and PtdIns/*myo*-inositol exchange enzymes previously characterized [7], significant amounts of [^{3}H]inositol can be incorporated into PtdIns. In the presence of ATP, the ghost-associated PtdIns 4-kinase and PtdIns4*P* 5-kinase activities rapidly phosphorylate the pool of labelled PtdIns. The polyphosphoinositides (but not the PtdIns) labelled in this way are susceptible to hydrolysis by the agonist- and G-protein-regulated PLC present in the ghosts. Ghosts labelled *in vitro* are at least as responsive to agonist and guanine nucleotide stimulation of PLC as are ghosts prepared by hypo-osmotic lysis of [^{3}H]inositol-labelled intact erythrocytes. Since the anatomically simple turkey erythrocyte ghosts contain little intracellular membrane, and are fully depleted of cytosolic components (e.g. lipid carrier proteins), these results provide good evidence for independent synthesis of hormone-sensitive phosphoinositide pools in the plasma membrane, as has been proposed for GH_3 cells [15]. However, it will be important to verify this in plasma-membrane preparations from more complex cells which contain more extensively developed endoplasmic reticulum.

Isoprenaline was found to increase ADP[S]+GTP- or ADP[S]+GTP[S]-stimulated PLC activity in ghost preparations labelled with [^{3}H]inositol *in vitro*. Stimulation of PLC by isoprenaline also occurred in the absence of the P_{2y}-purinergic agonist, but exhibited an absolute requirement for guanine nucleotides (GTP or GTP[S]). AlF_4^--stimulated PLC activity was not modified by isoprenaline. The EC_{50} for the isoprenaline-stimulated PLC activity (126 nM) is similar to EC_{50} values for activation of turkey erythrocyte adenylate cyclase by isoproterenol (C. Vaziri & T. K. Harden, unpublished work). These results suggest that a population of β-adrenergic receptors directly activates a G-protein linked to PLC in turkey erythrocytes.

Rooney *et al.* [14], who independently observed that isoprenaline activates PLC in turkey erythrocyte membranes, have shown that the α-adrenergic antagonists yohimbine and prazosin were without effect on isoprenaline-stimulated PLC. As an extension of these findings, the stimulatory effect of isoprenaline on PLC activity was inhibited in a dose-dependent manner by a range of β-adrenergic-receptor antagonists. The K_i obtained for inhibition of isoprenaline-stimulated PLC activity by propranolol is comparable with values for inhibition of turkey erythrocyte adenylate cyclase (C. Vaziri & T. K. Harden, unpublished work; [16]). The pharmacological properties of the turkey erythrocyte β receptor differ from mammalian β1 or β2 receptors [11]. Although preliminary evidence indicates that isoprenaline-stimulated adenylate cyclase and PLC activities are inhibited with similar K_i values by a range of β1-, β2- and non-selective β-receptor antagonists (C. Vaziri & T. K. Harden, unpublished work), definitive evidence for the ability of a single β-receptor subtype to communicate with both G_s and G_{PLC} depends on the functional reconstitution of the purified receptor and G-protein components in phospholipid vesicles [17].

To explain the stimulatory effects of reconstituted G-protein βγ subunits on P_{2y}-agonist- and GTP-stimulated PLC activity in turkey erythrocyte ghosts, Boyer *et al.* [6] postulated that βγ dimers may interact with free α_{PLC} in the ghost membranes, and, by mass action, drive the formation of more $\alpha\beta\gamma_{PLC}$ heterotrimers capable of successful coupling with the activated P_{2y} receptor. We hypothesized that the presence of isoprenaline might cause selective dissociation of G_s heterotrimers coupled to β-adrenergic receptors, thereby causing an increase in the amount of free βγ subunits in the plasma membrane. As with exogenously added βγ subunits, these might interact with the excess α_{PLC} to increase the number of $\alpha\beta\gamma_{PLC}$ heterotrimers available for interaction with the purinergic receptor. As would be predicted by this model, isoprenaline did increase the Ins*P* response produced by ADP[S] and GTP. However, the β-agonist also enhanced PLC activity in the absence of the purinergic agonist, if GTP or GTP[S] was present. This indicates that the effect of isoprenaline was not to promote efficient coupling between activated P_{2y} receptors and G_{PLC}. In support of this, stimulation of adenosine receptors, which also couple to G_s in turkey erythrocyte membranes [10], did not affect guanine-nucleotide- or guanine-nucleotide+purinergic-agonist-activated PLC, demonstrating that the stimulatory effect of isoprenaline was independent of G_s activation. Furthermore, Rooney *et al.* [14] have shown that the mechanism by which isoprenaline stimulates PLC is cholera-toxin-insensitive, and is therefore unlikely to be G_s-dependent. Addition of cyclic AMP or its cell-penetrating analogue dibutyryl cyclic AMP did not affect PLC activity under basal or agonist/guanine-nucleotide-stimulated conditions, indicating

that adenylate cyclase activation is not necessary for isoprenaline-stimulated PLC activity.

The ability of isoprenaline to shorten the lag phase of PLC activation by GTP[S] alone is consistent with the idea that the β-adrenergic receptor directly couples to a G_{PLC}, and that the mechanism of G-protein activation involves accelerated guanine-nucleotide exchange [5]. The fact that the stimulatory effect of maximally effective doses of P_{2y}- and β-receptor agonists is partially additive differs from the results reported by Rooney *et al.* [14], who found no additivity at maximal doses of P_{2y}-receptor and β-adrenergic-receptor agonists. The reason for this discrepancy is not known. Nevertheless, since in neither case was full additivity observed, it is possible that common components are involved in both β-adrenergic- and P_{2y}-receptor-stimulated PLC responses, and that the availability of either the PLC or the relevant G-protein is limiting at high agonist doses.

It will be important to determine definitively whether the turkey erythrocyte β-adrenergic receptor and the purinergic receptor both activate PLC via the same G-protein. One possible approach is to measure agonist-stimulated GTP[^{35}S] or [α-^{32}P]GTP binding to purified G-protein when reconstituted with purified receptors in phospholipid vesicles [17]. It is likely that the G-protein(s) responsible will be of the $G_{q/11}$ [18] family recently shown to activate PLCβ isoenzymes [3,4]. An AlF_4^- dependent PLC-stimulating activity purified from cholate extracts of turkey erythrocyte membranes contains a predominant 43 kDa protein recognized by antisera to a *C*-terminus region peptide common to $G_{q/11}$ [2]. It remains possible, however, that turkey erythrocyte membranes contain distinct G-proteins, which mediate PLC activation by different hormone receptors.

It was decided to determine whether the same molecular species of PLC was regulated by both purinergic and β-adrenergic stimuli. A 150 kDa PLC purified from turkey erythrocyte cytosol reconstitutes with 'uncoupled' turkey erythrocyte ghosts in an ADP[S]- or isoprenaline-regulated manner (when GTP[S] is present). The isoprenaline dose-dependency of reconstituted PLC activity is similar to that observed for isoprenaline stimulation of maximally responsive ghosts which have been prepared in the presence of Mg^{2+}. These results demonstrate that a single species of PLC effector enzyme can be regulated by P_{2y}-purinergic or β-adrenergic agonists.

Ghosts prepared from ADP[S]-pretreated [^{3}H]inositol-labelled erythrocytes exhibited decreased ability to respond to purinergic agonist + GTP[S] (13). However, PLC responses to GTP[S] or to GTP[S] + isoprenaline were unimpaired, indicating that G-protein/PLC coupling was unaffected in response to ADP[S] pretreatment, and that homologous desensitization [19] occurred at the level of P_{2y}-receptor/G-protein interaction. In contrast, when cells were pretreated with isoprenaline, the PLC responsiveness of subsequently prepared ghosts to both β-adrenergic and purinergic agonists was diminished (heterologous desensitization [19]), although G-protein/PLC coupling was unimpaired.

On the basis of experiments with phorbol esters which activate protein kinase C, Martin & Harden [13] suggested that desensitization of the purinergic receptor after stimulation of intact cells with purinergic agonists, is, at least in part, mediated by protein kinase C [13]. It is perhaps not surprising that stimulation of the β-adrenergic receptor causes desensitization of the purinergic receptor, since stimulation of both receptors results in generation of diacylglycerol (and $InsP_3$), and therefore in activation of common downstream effectors (e.g. protein kinase C). We have not investigated the mechanism of desensitization of the PLC-coupled β-adrenergic receptor, although preliminary experiments indicate that pretreatment of intact cells with dibutyryl cyclic AMP (without or in combination with ADP[S]) cannot induce desensitization of the β-adrenergic-receptor-stimulated PLC in subsequently prepared ghosts. It is possible that desensitization of the isoprenaline-stimulated PLC response is due to the β-adrenergic-receptor kinase, which phosphorylates the agonist-occupied form of the β-adrenergic receptor, and impairs its ability to communicate with G-protein [20].

The results demonstrate that β-adrenergic stimuli can elicit a PLC response as well as adenylate cyclase activation in the same cell. It is possible, but not proved by these results, that both β-adrenergic receptors and P_{2y} receptors converge on a common G-protein to stimulate a single species of PLC. Further work is therefore required to identify the subtype of β-adrenergic receptor and the species of G-protein(s) which mediate the isoprenaline-stimulated PLC activity in turkey erythrocytes.

We are grateful to A. L. Willcocks for sending us β-adrenergic-receptor antagonists, and to A. Thomas for communicating results before publication. We thank A. J. Morris, G. Waldo and T. K. Harden for purified PLC. Some of the experiments described were performed in T. K. Harden's laboratory in the Pharmacology Department, University of North Carolina, Chapel Hill, NC, U.S.A. C.V. is supported by an M.R.C. studentship.

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Received 23 September 1991/10 January 1992; accepted 22 January 1992