Stimulation of high-affinity GTPase activity and cholera toxin-catalysed [^{32}P]ADP-ribosylation of G_i by lysophosphatidic acid (LPA) in wild-type and α2C10 adrenoceptor-transfected Rat 1 fibroblasts

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Lysophosphatidic acid (LPA) stimulated high-affinity GTPase activity in membranes of Rat 1 fibroblasts. This effect was dose-dependent, with maximal effects at 10 μM LPA, and was attenuated by pertussis toxin but not by cholera toxin pretreatment of the cells, indicating that the effect was likely to be produced by a G_i-like G-protein. LPA stimulation of high-affinity GTPase was also observed in a clone of Rat 1 fibroblasts that had been transfected to express the human α2C10 adrenoceptor. The α2 adrenoceptor agonist UK14304 also stimulated high-affinity GTPase activity in membranes of these cells, but not in parental Rat 1 cells. LPA was also able to promote cholera toxin-catalysed [^{32}P]ADP-ribosylation of G_i. This effect of LPA was also prevented by pretreatment of the cells with pertussis toxin but not cholera toxin. LPA-stimulated cholera toxin-catalysed [^{32}P]ADP-ribosylation of G_i in membranes of the α2C10 adrenoceptor-expressing clone was additive with that produced by UK14304. Dose–response curves for LPA in the two assays of G-protein activation were coincident. The results presented herein demonstrate conclusively that the pertussis toxin-sensitive effects of LPA in Rat 1 fibroblasts and a clone of these cells expressing the α2C10 adrenoceptor are produced directly by the activation of G_i.

INTRODUCTION

Lysophosphatidic acid (LPA) acts as a mitogen for many cells [1,2]. Despite observations of LPA-induced transient increases in intracellular [Ca^{2+}] [3], generation of inositol phosphates [4] and phospholipase D stimulation of phosphatidylcholine breakdown [5], there have been reservations, partly because of the difficulties in performing classical saturation binding experiments resulting from the relative hydrophobicity of the compound, as to whether or not LPA functions via binding to a serpentine receptor and subsequent activation of a heterotrimeric G-protein (see [6], for review). Data in Rat 1 fibroblasts, which include synergistic regulation of LPA-induced Ins(1,4,5)P_3 generation by guanine nucleotides [4], enhanced GTP loading of p21^{ras} [7] and stimulation of the phosphorylation state of mitogen-activated protein (MAP)kinase [7] in a pertussis toxin-sensitive manner, are clearly indicative of such a mechanism. Yet LPA activation of G_i (or indeed of any other G-protein) has not been demonstrated directly.

We have recently demonstrated in a clone of Rat 1 fibroblasts transfected to express the human α2C10 adrenoceptor (which is pharmacologically equivalent to the α2A adrenoceptor) that agonist activation of this receptor can also cause enhanced GTP loading of p21^{ras}, phosphorylation of MAP kinase and enhanced incorporation of [^{3}H]thymidine in a pertussis toxin-sensitive manner [8]. We have also previously demonstrated in membranes from these cells that an α2 adrenoceptor agonist is able to stimulate the high-affinity GTPase activity of G_i [9,10] and allow cholera toxin-catalysed [^{32}P]ADP-ribosylation of this G-protein [11]. This second assay is dependent upon agonist-occupied receptor-induced activation of a G-protein and can thus be used to demonstrate directly the existence of receptor-linked G-protein [11].

In this report, we make use of these two approaches to show directly that LPA is able to activate G_i in both wild-type Rat 1 cells and in the α2C10 adrenoceptor-expressing clone. We further demonstrate that LPA activation of G_i in membranes of these cells is additive with that produced by agonist activation of the α2C10 adrenoceptor, and that these effects are produced at concentrations of LPA similar to those recorded for both LPA stimulation of Ins(1,4,5)P_3 generation [4] and phospholipase D-induced phosphatidylcholine hydrolysis [5] in Rat 1 fibroblasts. These result provide the first direct demonstration of LPA activation of G_i and further strengthen the argument that LPA functions via a G-protein-linked serpentine receptor.

MATERIALS AND METHODS

Materials

Reagents were obtained from the following sources: [^{3}H]-yohimbine (80 Ci/mmol) and [γ-^{32}P]GTP, Amersham International; [^{32}P]NAD$^+$ (800 Ci/mmol), Dupont/New England Nuclear; pertussis toxin, Porton Products, Porton Down, Wiltshire, U.K; cholera toxin and LPA, Sigma; all materials for tissue culture, Gibco.

Expression of recombinant DNA encoding the α2C10 adrenoceptor

Stable expression of genomic DNA corresponding to the human platelet α2C10 adrenoceptor (obtained from the American Tissue Type Collection, name of clone, HPalpha2 GEN) [12] was achieved using the mammalian expression vector pDOL [13] as previously described [11]. The cells used in this study, Rat 1 α2A 1C (1C) [9–11], expressed approx. 1.0 pmol/mg of membrane protein of the α2C10 adrenoceptor in the passages used, as

Abbreviations used: LPA, lysophosphatidic acid; MAP kinase, mitogen-activated protein kinase; FCS, fetal calf serum.
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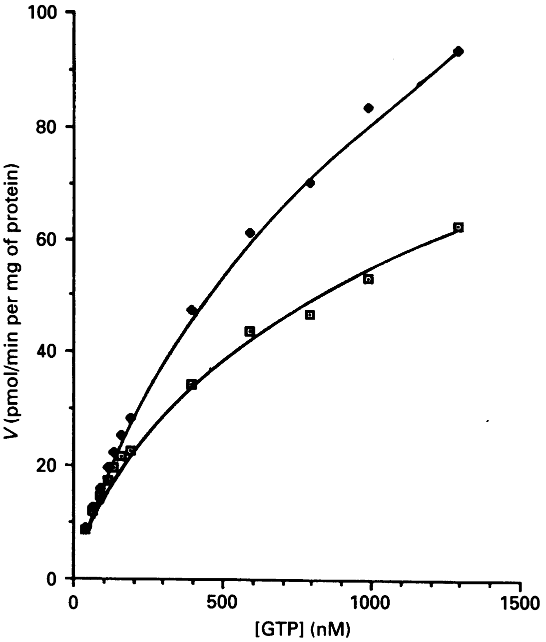

Figure 1 Characterization of enzymic characteristics of high-affinity GTPase activity in membranes of clone 1C cells

Values for high-affinity GTPase activity were calculated by subtracting hydrolysis by low-affinity GTPases and correcting for the specific activity of [γ-^{32}P]GTP at each concentration. Assays were performed in the presence (◆) or absence (□) of LPA (10 μM). Points represent means for quadruplicate assays; S.E.M.s were less than 7% of the mean for all points.

assessed by the specific binding of the α2 adrenoceptor antagonist [^{3}H]yohimbine (results not shown).

Cell culture

Rat 1 fibroblasts and cells of clone 1C were grown in Dulbecco's modified Eagle's medium supplemented with 5% (v/v) donor calf serum, penicillin (100 units/ml) and streptomycin (100 μg/ml) in 5% CO_2 at 37 °C. Cells were grown in 75 cm^2 tissue culture flasks and were harvested just before confluency. In a number of cases, cells were treated with either pertussis toxin (25 ng/ml) or cholera toxin (100 ng/ml) for 16 h before harvest. Membranes were prepared from the cells by homogenization with a teflon-on-glass homogenizer and differential centrifugation as described for a variety of other cells [14].

High-affinity GTPase activity measurements

These measurements were routinely performed as in [15] using [γ-^{32}P]GTP (0.5 μM, 60000 c.p.m.) and various concentrations of either LPA or UK14304. Non-specific (low-affinity) GTPase activity was assessed in parallel assays containing 100 μM GTP. In a number of experiments designed to measure enzymic parameters of the activity, concentrations of GTP were altered and specific activity subsequently corrected [14]. Estimates of $V_{max.}$ and K_m for GTP from such experiments were calculated by fitting the data using the LIGAND non-linear least-squares programme. As in other studies in which high-affinity GTPase has been assessed (e.g. [14]), basal activity (measured at 0.5 μM GTP) varied from experiment to experiment and from cell passage to passage within an approx. 2-fold range (20–40 pmol/min per mg of membrane protein).

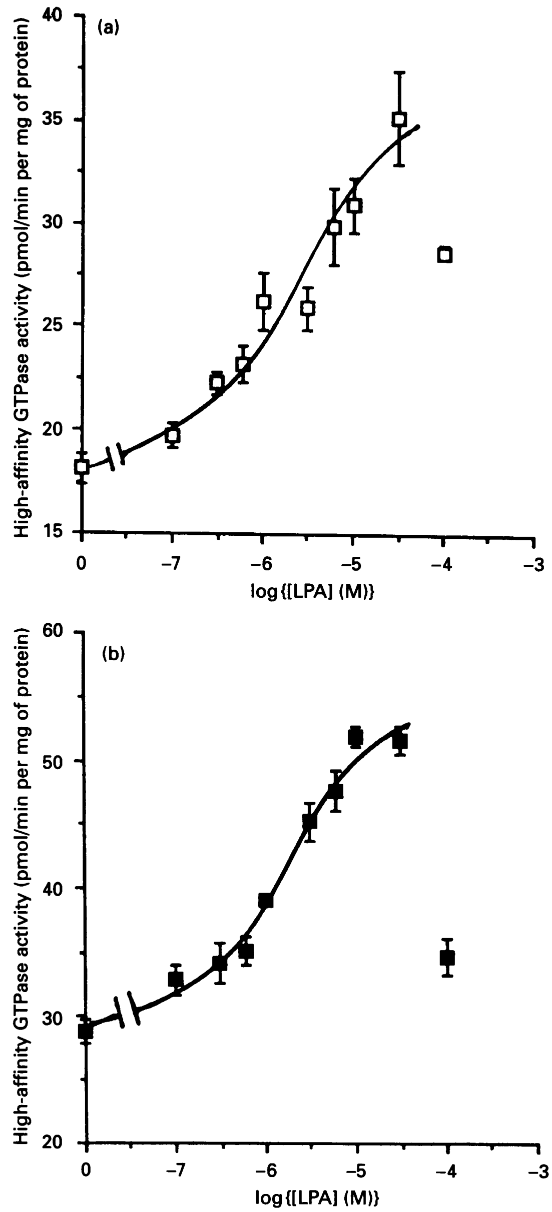

Figure 2 LPA stimulation of high-affinity GTPase activity in membranes from parental Rat 1 fibroblasts (□) and clone 1C cells (■)

The effect of varying concentrations of LPA on basal high-affinity GTPase activity (measured at 0.5 μM GTP) was measured in membranes of either parental Rat 1 fibroblasts (□) or clone 1C cells (■). Results represent means ± S.E.M. of quadruplicate assays derived from a single experiment representative of three. In all cases, the measured activity in the presence of 100 μM LPA was substantially lower than that recorded at 10–30 μM.

Cholera toxin-catalysed [^{32}P]ADP-ribosylation

[^{32}P]ADP-ribosylation of membranes of the cells used in these experiments was performed in the absence of added guanine nucleotide basically as described previously [11,16], except that sodium phosphate, pH 7.0, replaced potassium phosphate, pH 7.0. Further additions to the assays were as detailed in the Results section. Cholera toxin was used at a concentration of

Table 1 Basal high-affinity GTPase activity and its regulation by LPA or UK14304 in membranes of parental Rat 1 and Rat 1 clone 1C cells

For parental Rat 1 cells, statistically significant stimulation by LPA ($P < 0.02$ in each case) but not by UK14304 of the basal activity was noted for both control and cholera toxin-pretreated cells. No significant stimulation by either agonist was recorded in membranes from pertussis toxin-pretreated cells. Pertussis toxin pretreatment produced a statistically significant ($P = 0.009$) reduction in basal GTPase activity, as has been noted previously [16]. For Rat 1 clone 1C cells, statistically significant stimulation (LPA, $P < 0.05$ in each case; UK14304, $P < 0.001$ in each case) of the basal activity was noted for both LPA and UK14304 in membranes from both control and cholera toxin-pretreated cells but no significant stimulation by either agonist (LPA, $P = 0.57$; UK14304, $P = 0.22$) was recorded in membranes from pertussis toxin-pretreated cells. Pertussis toxin pretreatment produced a statistically significant ($P = 0.004$) reduction in basal GTPase activity, as has been noted previously [16]. For both cell types, values in brackets represent the fold stimulation of basal high-affinity GTPase activity by the agonists. Data, which are presented as means ± S.D., were derived from a single experiment representative of three performed on different membrane preparations.

		High-affinity GTPase activity (pmol/min per mg of membrane protein)		
Cell type	Pretreatment	Basal	+LPA (10 μM)	+UK14304 (10 μM)
Parental Rat 1	Control	22.9 ± 2.1	33.9 ± 4.1 (1.48)	19.2 ± 1.8
	Cholera toxin	21.8 ± 3.4	31.6 ± 1.1 (1.45)	19.3 ± 1.2
	Pertussis toxin	15.0 ± 2.1	12.8 ± 2.1	13.8 ± 1.9
Rat 1 clone 1C	Control	31.6 ± 4.6	45.7 ± 3.4 (1.45)	86.9 ± 1.6 (2.75)
	Cholera toxin	28.3 ± 5.5	39.1 ± 3.5 (1.38)	76.4 ± 2.8 (2.70)
	Pertussis toxin	14.1 ± 2.6	15.4 ± 2.5 (1.09)	17.4 ± 1.8 (1.24)

50 μg/ml. Analysis of the incorporation of radioactivity into polypeptides which were labelled in a toxin-specific manner was performed by scanning autoradiograms produced following SDS/PAGE of the samples with a Shimadzu gel scanner.

Binding experiments

Binding was assayed at 25 °C for 30 min in 10 mM Tris/HCl, 50 mM sucrose, 20 mM $MgCl_2$, pH 7.5 (buffer A) using [^{3}H]yohimbine (5 nM). Non-specific binding was defined in all cases by parallel assays containing 100 μM noradrenaline. Measured specific binding was corrected for receptor occupancy using the formalism of Cheng and Prusoff [16a] using a measured K_d for [^{3}H]yohimbine for these membranes of 0.5 nM (see [11]). Binding experiments were terminated by rapid filtration through Whatman GF/C filters followed by three washes of the filter with ice-cold buffer A (5 ml).

RESULTS

In membranes of clone 1C (a clone of Rat 1 cells transfected to express the α2C10 adrenoceptor), LPA stimulated high-affinity GTPase activity. In contrast, LPA had no effect on low-affinity GTPase activity (defined by the presence of 100 μM GTP). Basal high-affinity GTPase activity in membranes of these cells was characterized by an apparent K_m for GTP of 0.25 ± 0.03 μM and a $V_{max.}$ of 65.2 ± 4.3 pmol/min per mg membrane protein (Figure 1). Addition of 10 μM LPA increased $V_{max.}$ to 118.9 ± 8.4 pmol/min per mg membrane protein and also increased the apparent K_m for GTP to 0.45 ± 0.03 μM (mean ± S.E.M., $n = 3$) (Figure 1). LPA stimulation of high-affinity GTPase activity (measured at 0.5 μM GTP) was dose-dependent with an apparent EC_{50} of 1.0 ± 0.2 μM (mean ± S.E.M., $n = 3$) (Figures 2a and 2b). This was impossible to assess definitively, however, as at concentrations above 10–30 μM, a distinct reduction in the agonist-induced stimulation of high-affinity GTPase activity was noted routinely (Figure 2). As has previously been demonstrated [8–10], the α2 adrenoceptor agonist UK14304 also stimulated high-affinity GTPase activity in membranes of these cells, with $EC_{50} = 30$ nM. The stimulation of high-affinity GTPase produced by a maximally effective concentration of UK14304 in membranes of clone 1C cells was routinely some 3–4-fold greater than that produced by LPA (Table 1). UK14304 was unable, however, to stimulate high-affinity GTPase activity in membranes of parental Rat 1 cells (Table 1) whereas LPA also stimulated high-affinity GTPase activity in membranes of the parental cells in a similar dose-dependent manner (Figure 2). Maximal stimulation was obtained in the presence of approx. 10 μM LPA with an apparent EC_{50} of approx. 1.8 ± 0.3 μM (mean ± S.E.M., $n = 3$) but, as noted above, it was not possible to obtain a reliable estimate for this as concentrations of LPA above 30 μM produced a lower stimulation (Figure 2a). Pretreatment of either parental Rat 1 fibroblasts or cells of clone 1C with cholera toxin (100 ng/ml, 16 h) had no significant effect on either the basal high-affinity GTPase activity or the degree of stimulation achieved either by LPA in both cell lines or by UK14304 in membranes of clone 1C cells (Table 1). In contrast, pertussis toxin pretreatment (25 ng/ml, 16 h) of both of the cell lines significantly reduced the basal high-affinity GTPase activity (Table 1), as has been recorded previously [17], and essentially abolished both LPA and UK14304 stimulation of this activity (Table 1). Such an effect of pertussis toxin is routinely interpreted to indicate interaction of the receptor for the ligand with G_i [18].

When cholera toxin-catalysed [^{32}P]ADP-ribosylation was performed on membranes of clone 1C cells in the absence of added GTP, radioactivity was incorporated into two polypeptides of 45 and 42 kDa, which could be shown by comigration with *Escherichia coli*-expressed recombinant proteins (results not shown, but see [19]) to represent the long and short splice variants of $G_s\alpha$. In some cases, a polypeptide of 40 kDa also incorporated radiolabel very weakly under these conditions. Addition of UK14304 resulted in substantially higher levels of incorporation of [^{32}P]ADP-ribose into the 40 kDa polypeptide (Figures 3a and 3b), which has previously been shown to be the α subunit of G_i [11]. Addition of LPA also promoted incorporation of [^{32}P]ADP-ribose into this polypeptide (Figures 3a and 3b), and addition of both UK14304 and LPA produced essentially additive cholera toxin-catalysed ADP-ribosylation of G_i (Figure 4). Pretreatment of cells of clone 1C with cholera toxin resulted in a great reduction in incorporation of [^{32}P]ADP-ribose into the splice variants of G_s but it did not prevent UK14304, LPA or the combination of the two agents from inducing stimulation of cholera toxin-catalysed [^{32}P]ADP-ribosylation of G_i (Figures 3b and 4). Pretreatment of the cells with cholera toxin had the distinct practical advantage of making

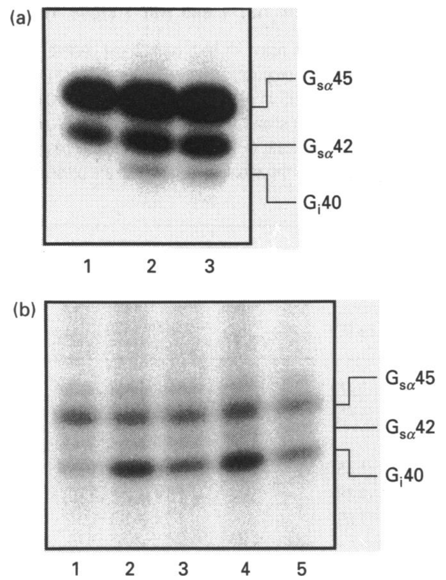

Figure 3 LPA and UK14304 both stimulate cholera toxin-catalysed [^{32}P]ADP-ribosylation of G_i in membranes of the α2C10 adrenoceptor-expressing clone 1C cells

Membranes (50 μg) of (**a**) untreated or (**b**) cholera toxin-pretreated (100 ng/ml, 16 h) clone 1C cells were incubated with cholera toxin and [^{32}P]NAD$^+$ in the absence of guanine nucleotides as described in the Materials and methods section. Samples were precipitated and resolved by SDS/PAGE and subsequently autoradiographed. Lane 1, [^{32}P]ADP-ribosylation in the absence of ligand; lane 2 (and lane 4 in **b**), +1 μM UK14304; lane 3 (and lane 5 in **b**) +10 μM LPA. The apparent molecular masses (in kDa) of both the long (45) and short (42) isoforms of $G_{s\alpha}$ and of G_i40 (40) are provided for reference.

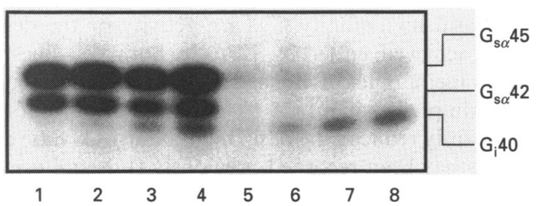

Figure 4 LPA and UK14304 additively stimulate cholera toxin-catalysed [^{32}P]ADP-ribosylation of G_i in membranes of clone 1C cells

Membranes (50 μg) of either untreated (lanes 1–4) or cholera toxin-pretreated (100 ng/ml, 16 h) (lanes 5–8) clone 1C cells were incubated with cholera toxin and [^{32}P]NAD$^+$ in the absence of guanine nucleotides as described in the Materials and methods section. Samples were precipitated and resolved by SDS/PAGE and subsequently autoradiographed. Lanes 1 and 5, no ligand; lanes 2 and 6, +10 μM LPA; lanes 3 and 7, +1 μM UK14304; lanes 4 and 8, +10 μM LPA +1 μM UK14304. The apparent molecular masses (in kDa) of both the long (45) and short (42) isoforms of $G_{s\alpha}$ and of G_i40 (40) are provided for reference.

receptor-regulated G_i the predominant substrate for cholera toxin-catalysed [^{32}P]ADP-ribosylation in these membranes (Figures 3b and 4). This allowed gels to be autoradiographed appropriately without 'blooming' from the large cholera toxin-induced incorporation of [^{32}P]ADP-ribose into G_s noted in untreated cells (Figures 3a and 4) interfering with visualization of the radioactivity incorporated into G_i. LPA stimulated cholera toxin-catalysed [^{32}P]ADP-ribosylation of G_i in membranes of cholera toxin-pretreated 1C cells dose-dependently with an apparent EC_{50} of approx. 1 μM (results not shown).

Pertussis toxin pretreatment of parental Rat 1 and clone 1C cells, which prevented subsequent pertussis toxin-catalysed [^{32}P]ADP-ribosylation of G_i (results not shown), prevented both LPA and UK14304 stimulation of cholera toxin-catalysed [^{32}P]ADP-ribosylation of this G-protein (results not shown, but see [11]).

DISCUSSION

LPA has been widely examined as a mitogen for many cell types [2,6]. This phospholipid has been demonstrated to be released from cells [20] and it is believed to act at a G-protein-linked receptor [21], activating a variety of intracellular signalling cascades including stimulation of inositol phosphate generation by a phosphoinositidase C [1,4] and GTP loading of p21^{ras} [7], which leads subsequently to the phosphorylation and activation of MAP kinase [7]. The putative LPA receptor has not been isolated as either a protein or a corresponding cDNA clone but cross-linking studies with an LPA analogue have identified a 38 kDa polypeptide which may represent the receptor [21]. Many [6,7], but not all [5], of the responses to LPA in Rat 1 fibroblasts are attenuated by pretreatment of cells with pertussis toxin. This is taken to imply that the binding of LPA to a receptor results in the activation of G_i [18]. Yet despite the synergistic effect of guanine nucleotides on LPA-stimulated generation of inositol phosphates [1,4], little direct evidence has demonstrated activation of a G-protein by LPA.

In this report, we demonstrate clearly and directly by two complementary approaches that LPA leads to the activation of G_i in Rat 1 fibroblasts and a clone derived from these cells which expresses stably the α2C10 adrenoceptor [9–11]. A classical approach for the demonstration of G-protein activation by a receptor ligand is agonist stimulation of high-affinity GTPase activity in cellular membranes [18]. The cells transfected to express the α2C10 adrenoceptor provided a positive control for the studies with LPA because α2 adrenoceptors classically couple to G_i and, as noted above, in these cells display many signalling features in common with LPA, including GTP loading of p21^{ras} [8] and stimulation of the phosphorylation state of MAP kinase [8]. The selective α2 adrenoceptor agonist UK14304 stimulated high-affinity GTPase activity in membranes of clone 1C but not in parental Rat 1 cell membranes whereas LPA stimulated this activity in membranes from both cell lines (Table 1). The effect of LPA was demonstrated to result from an increase in $V_{max.}$ of the high-affinity GTPase activity but we also noted that the agonist produces a small increase in K_m for GTP (Figure 1). A similar pattern has been noted previously for fetal calf serum (FCS) stimulation of a pertussis toxin-sensitive high-affinity GTPase activity in membranes of C6 glioma cells [14]. It remains to be ascertained whether or not the previously recorded effects of FCS are actually a reflection of the presence of LPA within the serum, as the phospholipid is known to bind tightly to serum albumin [20]. The effect of LPA on high-affinity GTPase in clone 1C was lower than that produced by UK14304 (Table 1), which may reflect the relative levels of expression of the two receptors. Pertussis toxin pretreatment (100 ng/ml, 16 h) abolished the stimulatory effects of both LPA and UK14304 on high-affinity GTPase activity (Table 1); however, as noted above, not all effects of LPA in Rat 1 fibroblasts are attenuated by pertussis toxin treatment, e.g. stimulation of phospholipase D-mediated hydrolysis of phosphatidylcholine [5]. If this effect were to occur only after G-protein activation it would imply that the relevant G-protein would be pertussis toxin-insensitive and thus likely to be a member of the G_q family. Our data do not provide evidence for LPA activation of a pertussis toxin-insensitive G-protein, but they do not absolutely exclude the possibility. In practice, it is often difficult or impossible to measure stimulation of high-affinity GTPase activity above basal activity in response to agonist at receptors which are known from other experiments to couple to pertussis toxin-insensitive G-proteins [18]. Potential reasons for this have been discussed and may include the low intrinsic catalytic activity and low levels of expression of these G-proteins compared with pertussis toxin-sensitive G-proteins [18].

We and others have previously shown that addition of an agonist for a G_i-linked receptor to membranes which are maintained in the absence but not the presence of GTP can result in the [^{32}P]ADP-ribosylation of G_i by cholera toxin [11,16,22–24]. This is not the widely established function of cholera toxin, which is to catalyse the ADP-ribosylation of forms of G_s [18]. However, because observation of cholera toxin-catalysed modification of this polypeptide requires the addition of an agonist at a receptor that couples to $G_i\alpha$, it provides an elegant and clear demonstration of agonist-stimulated activation of G_i [18]. As with high-affinity GTPase activity, both LPA and UK14304 were able to produce incorporation of [^{32}P]ADP-ribose in membranes of the α2C10 adrenoceptor-expressing cells and the effect of the two agonists was additive (Figure 4). For both high-affinity GTPase activity and agonist-stimulation of cholera toxin-catalysed [^{32}P]ADP-ribosylation of G_i, the effect of LPA was dose-dependent and close to that recorded previously in Rat 1 cells for both LPA-stimulated generation of inositol phosphates and phospholipase D-mediated hydrolysis of phosphatidylcholine [4,5]. A range of other effects of LPA with rather different dose-effect curves has been reported. For example, GTP loading of p21^{ras} in Rat 1 cells has been noted to occur with an EC_{50} of approx. 20–30 nM [7] whereas stimulation of long-term mitogenesis requires micromolar concentrations [1]. It remains to be established to what extent such variations in potency of LPA in different assays might result from the activation of distinct forms of the LPA receptor, from variations in the subclones of cells used by different investigators, from difficulties associated with the solution and dilution of LPA, or from potential differences in receptor reserve for the various actions of this phospholipid.

One potential consideration in this study was that the effect of LPA in allowing cholera toxin to catalyse [^{32}P]ADP-ribosylation of $G_i\alpha$ as well as the more normal modification of $G_s\alpha$ might be related to a detergent-like effect of the lysophospholipid. To attempt to address this point, we performed cholera toxin-catalysed [^{32}P]ADP-ribosylations on membranes of clone 1C cells in the absence and presence of UK14304 (1 μM) with additionally the presence of a range of concentrations (up to 0.1 % v/v; approx. 3.5 mM) of SDS. This exhibited no ability to either allow agonist-independent cholera toxin-catalysed [^{32}P]ADP-ribosylation of G_i or modify the ability of the G_i-linked receptor agonist to produce this effect. Thus LPA-induced cholera toxin-mediated [^{32}P]ADP-ribosylation of G_i was shown not to be an artefact of a detergent-like property of the lysophospholipid.

The pertussis toxin-mediated attenuation of LPA function and the fact that expression of a constitutively activated form of $G_i2\alpha$ in Rat 1 fibroblasts is able to mimic many of the actions of LPA [25,26] imply that LPA, and by extension the receptor for LPA, interacts with G_i. The data herein provide the first clear demonstration that this is indeed the case.

Received 19 August 1993/16 September 1993; accepted 6 October 1993

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