FEBS Letters 534 (2003) 164–168 FEBS 26876

Modulation of phosphoinositide 3-kinase activation by cholesterol level suggests a novel positive role for lipid rafts in lysophosphatidic acid signalling

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Received 25 September 2002; revised 15 November 2002; accepted 6 December 2002

First published online 17 December 2002

Edited by Felix Wieland

Abstract Methyl-β-cyclodextrin (MβCD) was used to explore a role for cholesterol-enriched plasma membrane microdomains in coupling lysophosphatidic acid (LPA) stimulation to phosphoinositide 3-kinase (PI3K) activation. Cholesterol depletion strongly inhibited the production of phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate in Vero cells stimulated with LPA. In agreement, the phosphorylation of Akt/protein kinase B, but not of Erk kinases, was suppressed by MβCD. MβCD did not interfere with the overall phospholipid metabolism, and its effects were reversed in cholesterol add-back experiments. Finally, PI3K was detected in lipid rafts prepared from control but not MβCD-treated cells, suggesting that these microdomains contribute to LPA signalling by compartmentalising component(s) of the PI3K pathway.

Key words: Lysophosphatidic acid; Phosphoinositide 3-kinase; Lipid raft; Cholesterol depletion

1. Introduction

Lysophosphatidic acid (LPA), a lipid mediator involved in various normal and pathophysiological processes, promotes the proliferation or survival of numerous cell types, like a peptide growth factor. This biological activity is mediated by at least three different G protein-coupled receptors that can activate several signalling pathways involved in cell growth control. Besides the so-called Ras/mitogen-activated protein kinase pathway (MAPK), we and others have shown that phosphoinositide 3-kinase (PI3K) is an important intermediate of LPA mitogenic activity (review in [1]). PI3K phosphorylates the 3′ position of the inositol ring of phosphoinositides to produce lipids that are key actors of signalling pathways. For example, stimulation of fibroblast cells with LPA or other mitogens leads to the conversion of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) to phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3). This latter molecule controls the intracellular distribution and/or activation of signalling proteins involved in cell proliferation or survival, including the serine/threonine kinase Akt/protein kinase B (PKB) [2].

From a molecular point of view, two types of PI3Ks can be activated in response to LPA stimulation. PI3Kγ has the unique biochemical feature to be directly activated by heterotrimeric G proteins and was thus initially considered as the best candidate target of LPA [3]. On the other hand, we have shown that the PI3Kβ isoform was important for the mitogenic activity of LPA in non-haematopoietic cells that do not express PI3Kγ [4,5]. However, the mechanisms coupling LPA stimulation to PI3Kβ have to be further defined. Indeed, this isoform is classically activated downstream of tyrosine kinases through the recruitment of its associated p85 regulatory subunit to phosphotyrosine-containing motifs. Transactivation of the epidermal growth factor receptor (EGFR), as well as G protein βγ subunits, is thought to play an important role in PI3Kβ activation [6,7], but the molecular determinants of these processes have remained elusive.

Recently, plasma membrane microdomains have been proposed as initiation platforms for signalling pathways [8]. For example, detergent-resistant membranes enriched in glycosphingolipids and cholesterol are thought to play an essential role in T cell receptor activation by concentrating membrane-associated signalling proteins, thereby facilitating early signal transduction [9]. These so-called lipid rafts can be isolated from cell lysates by flotation on sucrose gradient. In epithelial or mesenchymal cells, lipid rafts called caveolae are characterised by the presence of caveolin, a cholesterol-binding protein that contains a scaffolding domain and tyrosine residues that can bind to a number of signalling molecules, including the EGFR [10]. Entire signalling modules such as the Ras/MAPK pathway have been identified in caveolae, suggesting that it might be an important compartment for growth factor-induced mitogenesis [11]. However, a number of recent reports indicate that these structures are in fact modulatory units for cell responses by sequestering signalling proteins (reviews in [8,10]). As an illustration of this, targeted downregulation of caveolin-1 in NIH-3T3 cells has been reported to lead to the hyperactivation of the Ras/MAPK pathway and to cell transformation [12].

Nevertheless, the dynamic properties of lipid rafts suggested that they might be important centres of early signalling processes. Because the mechanisms underlying PI3K activation by LPA in non-haematopoietic cells remain poorly under-

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Abbreviations: EGFR, epidermal growth factor receptor; LPA, lysophosphatidic acid; MAPK, mitogen-activated protein kinase; MβCD, methyl-β-cyclodextrin; PtdIns(3,4)P_2, phosphatidylinositol 3,4-bisphosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; PtdIns(3,4,5)P_2, phosphatidylinositol 3,4,5-trisphosphate; PKB, protein kinase B

0014-5793/02/$22.00
doi:10.1016/S0014-5793(02)03832-2

stood, we have explored whether lipid rafts could participate in this process.

2. Materials and methods

2.1. Materials

LPA and monoclonal anti-phosphoErk antibody were from Sigma. Polyclonal antibodies against phosphoAkt/PKB (Ser473) and against p85 were from Cell Signaling and Upstate Biotechnology, respectively. Monoclonal anti-caveolin antibody was from Transduction Laboratory. Cell culture reagents were from Life Technologies.

2.2. Cell culture and stimulation

Vero cells (ATCC CCL 81) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 7.5% foetal bovine serum and antibiotics. For stimulation, subconfluent cells were incubated overnight in serum-free medium before stimulation with LPA (10 μM, 5 min).

2.3. Depletion, repletion and assay of cellular cholesterol

To remove cholesterol, serum-starved cells were incubated in DMEM containing the indicated concentration of methyl-β-cyclodextrin (MβCD) (Sigma) for 1 h at 37°C. For cholesterol repletion, depleted cells were incubated with a solution containing 0.2 mM of preformed MβCD/cholesterol (10:1 mol/mol) complexes, similar to a previously described procedure [13]. To assay the cholesterol cell content, cellular lipids were extracted by the method of Bligh and Dyer [14] in the presence of 15 μg stigmasterol (Sigma) as an internal standard, then injected into a gas chromatograph, as previously described [15].

2.4. Phospholipid analysis

Cells grown in 10-cm plates were serum-starved for 24 h upon reaching 80–90% confluence, then labelled for 5 h with 0.2 mCi of [^{32}P]H_3PO_4 (Amersham) per ml in phosphate-free DMEM (Sigma). Cells were then processed or not for cholesterol depletion/repletion as indicated, followed by lipid extraction as previously described [7]. Lipid extracts were resolved by thin layer chromatography (TLC). For analysis of PI3K lipid products, the area corresponding to polyphosphoinositides was scraped off, deacylated by 20% methylamine and analysed by high performance liquid chromatography (HPLC) [7].

2.5. Immunoblotting procedures

For immunoblotting analysis of cell lysates, cells were directly scraped off in electrophoresis sample buffer, then boiled and processed for immunoblotting using standard procedures. Blots were developed using chemiluminescence (Amersham Pharmacia Biotech).

2.6. Raft isolation

This procedure was performed essentially as described [16]. Briefly, cells were scraped in MES-buffered saline (MBS: 25 mM MES pH 6.5, 150 mM NaCl) supplemented with 1% Triton X-100, 2 mM phenylmethylsulphonyl fluoride and 10 μg/ml each of aprotinin and leupeptin. Lysates were obtained using a Dounce homogeniser, clarified by centrifugation, then adjusted to 40% sucrose and placed at the bottom of an ultracentrifuge tube. A step sucrose gradient (5, 10, 15, 20, 25, 30%) in MBS was then layered on top. Samples were then centrifuged for 16 h at 34 000×g. Fractions of 1 ml were collected, diluted 1/1 in MBS, then subjected to a 14 000×g centrifugation for 30 min. Pellets were resuspended and boiled in electrophoresis sample buffer, then processed for immunoblotting analysis.

3. Results

3.1. Cholesterol level specifically modulates PI3K activation in response to LPA

To explore the role of rafts in PI3K signalling we used a non-transformed monkey kidney cell line (Vero) that displays a strong increase of PI3K lipid products upon LPA stimulation [7]. Because cholesterol is a critical component of detergent-resistant microdomains, we investigated whether treatment with MβCD to remove cell cholesterol would affect PI3K activation in response to LPA. MβCD is a water-soluble cyclic saccharide that has been used as a raft-disrupting agent due to its high capacity for encapsulating cholesterol [17]. As shown in Fig. 1, treatment of Vero cells with 7.5 mM MβCD strongly reduced the formation of the two major PI3K lipid products induced by LPA, i.e. PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3. To determine whether adding back cholesterol to the cells would relieve this inhibition, depleted cells were further incubated with a MβCD/cholesterol mixture described as a cholesterol repletion solution [13]. As shown in Fig. 1, this treatment partially restored the production of the two PI3K lipid products. Taken together, these results suggest that cholesterol depletion in Vero cells interferes with PI3K activation in response to LPA.

To determine whether cholesterol depletion specifically modulated the PI3K signalling pathway or exerted an overall inhibitory effect on LPA signalling, we compared the activation of Akt/PKB, a downstream effector of PI3K, with that of the MAPK Erk. This was achieved by studying the phosphorylation status of both kinases in lysates of cells treated with MβCD and LPA, using immunoblotting with phosphospecific antibodies. As expected in light of the results in Fig. 1, MβCD strongly reduced Akt/PKB phosphorylation induced by LPA, the inhibition being total when the MβCD concentration reached 7.5 mM. Cholesterol repletion with the MβCD/cholesterol mixture partially relieved this inhibition (Fig. 2A). In contrast with Akt, Erk phosphorylation induced by LPA was much less altered by MβCD treatments (20–25% inhibition at the highest doses of MβCD, Fig. 2B). Moreover, immunoblotting of the p85 subunit of PI3K in lysates from control or MβCD-treated cells did not reveal any variation in p85

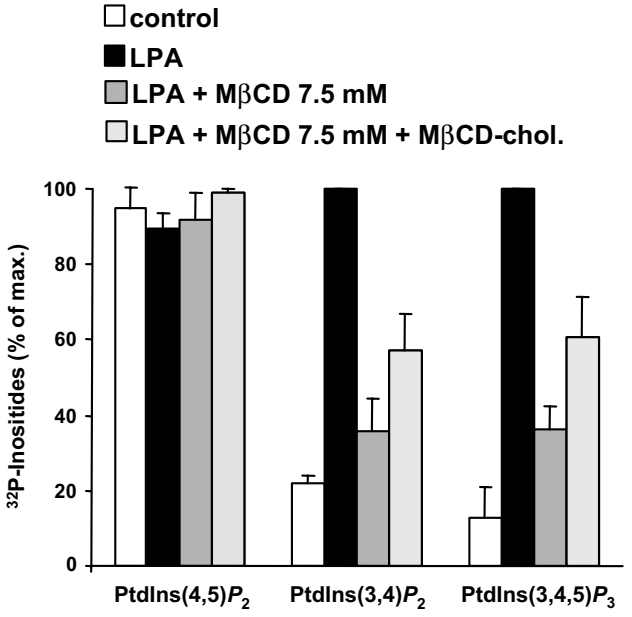

Fig. 1. Cholesterol level regulates the synthesis of PI3K lipid products induced by LPA. Serum-starved Vero cells were incubated for 5 h with [^{32}P]H_3PO_4, then stimulated or not with LPA as indicated. Lipids were then extracted, separated by TLC, and phosphoinositides were further analysed by HPLC. When indicated, cells were subjected before stimulation to cholesterol depletion using 7.5 mM MβCD (dark grey bars), followed by cholesterol repletion using a MβCD/cholesterol mixture (light grey bars). Data represent mean ± S.E.M. from three experiments.

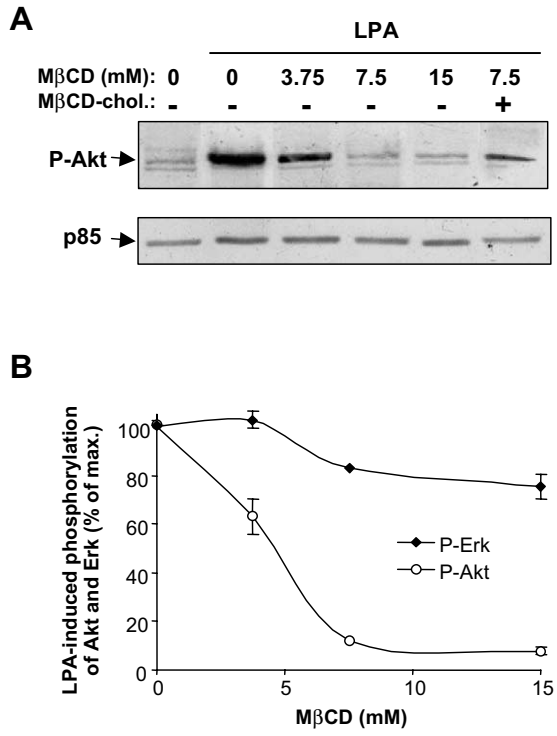

Fig. 2. Effect of cholesterol level modulation on LPA-induced phosphorylation of Akt and Erk. A: Quiescent Vero cells were treated for 1 h with the indicated concentration of MβCD, followed by cholesterol repletion with the MβCD/cholesterol mixture when indicated (MβCD-chol). Cells were then stimulated with LPA, lysed in SDS–PAGE sample buffer and processed for immunoblotting analysis against phosphoAkt (P-Akt), or p85 to control sample preparation and gel loading. B: Anti-phosphoErk immunoblotting was performed on cells processed as in A. Anti-phosphoAkt and anti-phosphoErk immunoblots were analysed by densitometry using the NIHimage software. Data represent mean ± S.E.M. from three experiments.

amounts, thereby excluding a major loss of integrity in cholesterol-depleted cells (Fig. 2A). In addition, because EGFR is a critical mediator of PI3K activation induced by LPA [7], we checked that MβCD treatments did not significantly modify LPA-induced EGFR phosphorylation, although cholesterol depletion moderately increased the basal phosphorylation of EGFR in unstimulated cells (data not shown). Altogether, these results suggest that cholesterol depletion specifically affects the PI3K pathway, implying a novel positive role for lipid rafts in LPA signalling.

3.2. *MβCD as a tool to manipulate cell cholesterol without interfering with the overall phospholipid metabolism*

Although the above data suggested that cholesterol depletion specifically altered PI3K signalling, it was important to check that MβCD treatments actually modified the level of cellular cholesterol, without interfering with the overall phospholipid metabolism. This was achieved first by measuring cholesterol content in control and MβCD-treated cells using gas chromatography. As shown in Fig. 3A, a 1 h treatment with MβCD dose-dependently removed cholesterol from Vero cells, the depletion reaching over 80% at the concentration of 7.5 mM. Under these conditions, a subsequent incubation with MβCD/cholesterol complexes restored the cellular cholesterol to nearly 80% of its initial level, thereby validating the use of MβCD and the MβCD/cholesterol mixture to manipulate cholesterol level. To determine whether these treatments affected the overall metabolism of phospholipids, cells were metabolically labelled with [^{32}P]H_3PO_4 followed by lipid extraction and TLC. Fig. 3B shows that treatment of Vero cells with MβCD, followed or not by the cholesterol repletion mixture, did not modify the labelling of major cellular phospholipids, including phosphoinositides. We conclude that MβCD allows manipulating cholesterol level without significantly interfering with the phospholipid metabolism.

3.3. *Cholesterol depletion disrupts lipid rafts and PI3K localisation*

Because cholesterol is an important component of detergent-resistant microdomains, the effect of cholesterol depletion on PI3K activation suggested an essential, yet unknown, positive role of lipid rafts in this pathway. To begin to explore this question, Triton X-100-resistant membranes were pre-

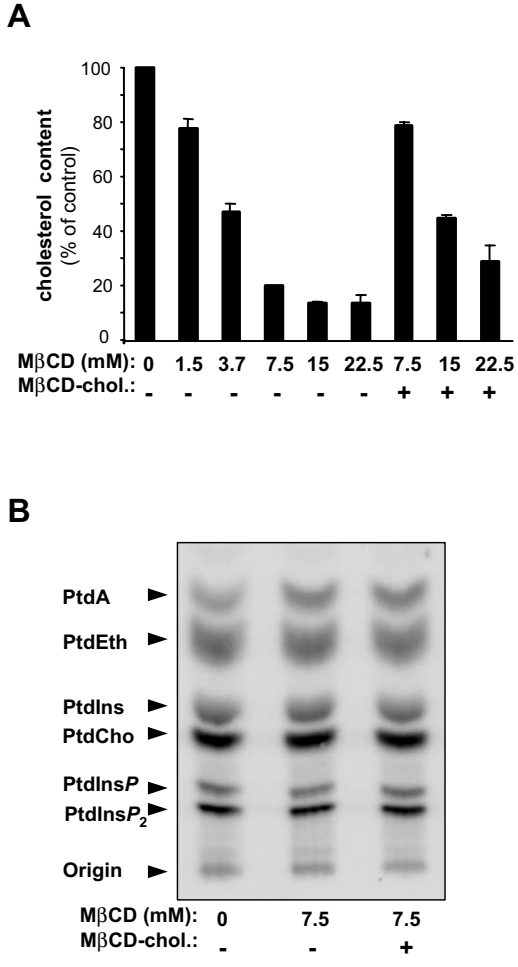

Fig. 3. Specificity of MβCD treatments regarding cellular cholesterol and phospholipid metabolism. A: Serum-starved Vero cells were treated for 1 h with the indicated concentration of MβCD followed by cholesterol repletion using MβCD/cholesterol complexes (MβCD-chol) when indicated. Cells were then subjected to lipid extraction, followed by cholesterol assay. B: Cells were incubated with [^{32}P]-H_3PO_4, then subjected or not to cholesterol depletion (7.5 mM MβCD) and repletion as indicated. Lipids were then extracted, separated by TLC, revealed with a phosphorimager and identified with reference to purified standards. PtdA, phosphatidic acid; PtdCho, phosphatidylcholine; PtdEth, phosphatidylethanolamine.

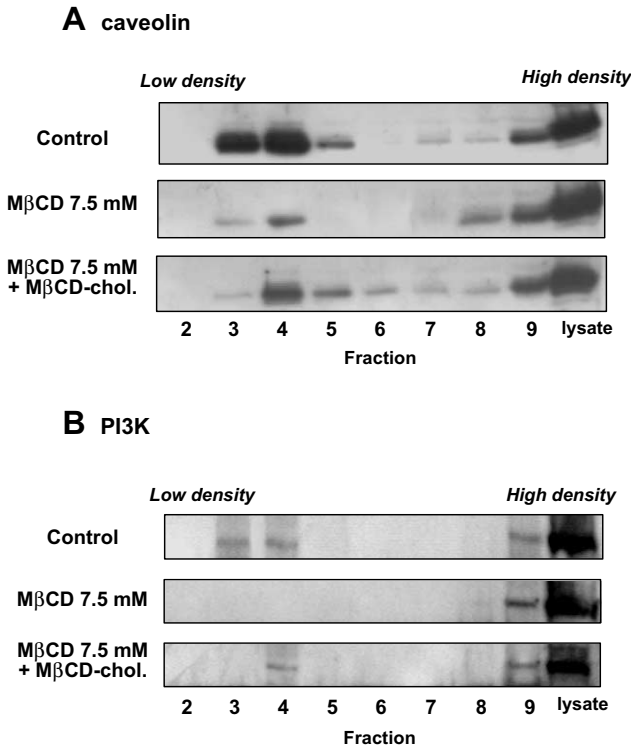

Fig. 4. Effect of cholesterol depletion on lipid rafts and distribution of the p85 PI3K subunit. Vero cells were treated without (control) or with 7.5 mM MβCD, followed by cholesterol repletion with the MβCD/cholesterol mixture when indicated (MβCD-chol). Cells were then lysed with 1% Triton X-100, followed by fractionation on sucrose step gradients. Fractions were collected then analysed by immunoblotting anti-caveolin (A) and anti-p85 subunits of PI3K (B).

pared from Vero cells using centrifugation across sucrose gradients. Upon macroscopic observation, a light-refractive band was clearly visible in the top third of gradients prepared from control cells (not shown), strongly suggesting the presence of lipid rafts in these fractions. The different density fractions were then collected and analysed by anti-caveolin immunoblotting. In agreement with the macroscopic observation, a large amount of caveolin was detected in fractions 3 and 4 that were thus taken as raft-containing fractions (Fig. 4A). When cells were treated with 7.5 mM MβCD, the intensity of the light-refractive band (not shown) and caveolin immunodetection in fractions 3 and 4 were both severely decreased, then restored by the cholesterol repletion mixture, albeit below its initial level (Fig. 4A). These results confirmed that cholesterol depletion with MβCD disrupted lipid rafts, therefore suggesting that the microdomain integrity is important for PI3K activation. Nevertheless, it was essential to identify the presence of PI3K in lipid rafts, which was achieved using immunoblotting. Fig. 4B shows that p85 can be detected in rafts from control, but not from cholesterol-depleted cells, and cholesterol repletion restored the association of p85 with lipid rafts. We propose that the regulation of PI3K activity by cholesterol level points to a novel positive function for lipid rafts in LPA signalling.

4. Discussion

Because the mechanisms coupling LPA stimulation to PI3K activation remain poorly understood, we searched for a participation of detergent-resistant membrane microdomains, putative regulatory platforms for proximal signalling events. Our approach was based on the disruption of these microdomains using the cholesterol-encapsulating drug MβCD, since cholesterol is an essential backbone component of these structures. Our assays of cell cholesterol in parallel with phospholipid analysis showed that MβCD turned out to be a very powerful tool to manipulate cholesterol level without seemingly interfering with the overall cell content and metabolism of phospholipids. In addition, we verified that MβCD treatment did alter the amount of lipid rafts present in Vero cells, which makes it possible to consider, in these conditions, the cholesterol content of cells as an indirect marker of microdomain integrity.

We thus observed that cholesterol level strongly modulated the activation of PI3K in response to LPA. Indeed, cholesterol depletion inhibited both LPA-stimulated synthesis of PI3K lipid products and Akt/PKB phosphorylation, whereas cholesterol add-back experiments restored both responses, although below their initial level. Cell fractionation results suggested that this partial effect of repletion is due to the conditions of incubation with MβCD/cholesterol complexes that did not allow cells to fully recover their initial content in lipid rafts.

Interestingly, cholesterol depletion was found to abolish Akt phosphorylation while its inhibitory effect on the synthesis of PI3K lipid products was not total. Still, MβCD had a strong effect (80% inhibition) on the production of PtdIns(3,4)P_2, a critical molecule for Akt activation [2]. Moreover, the current understanding of Akt activation mechanism seems compatible with the notion that this kinase could need a threshold concentration of PI3K lipid products to get activated. In brief, PI3K lipid products do not directly activate Akt but induce its membrane translocation and colocalisation with its upstream activating kinases, the phosphoinositide-dependent kinases (PDKs) that are also controlled by PI3K lipid products. It thus seems likely that a minimal concentration of these lipids must be achieved to induce a sufficient degree of 'clustering' of Akt and the PDKs required for Akt activation.

Regarding the specificity of cholesterol depletion on LPA signalling, we observed that the MAPK pathway was not strongly affected by MβCD. Indeed, only a moderate inhibition of Erk phosphorylation was observed at the highest doses of MβCD, as expected since MAPK activation in response to LPA is partly dependent on PI3Kβ in Vero cells [5]. In agreement, cholesterol depletion did not interfere with the LPA-induced phosphorylation of EGFR (data not shown), a critical mediator of MAPK and PI3K activation [7]. Consequently, this excluded the possibility that MβCD induced an overall loss of cell sensitivity to LPA by depleting, together with cholesterol, important membrane components of the cell signalling machinery, e.g. LPA receptors. These data rather suggest that cholesterol depletion specifically affects at least one component of the PI3K pathway.

Although a positive role for lipid rafts has been clearly established in lymphocyte signalling [8], these structures appeared to play rather a regulatory role in non-haematopoietic cells. For example, cholesterol depletion has been reported to hyperactivate the Ras/MAPK pathway in EGF-stimulated fibroblasts [18], which may be due to EGFR sequestration in lipid rafts leading to impaired ligand accessibility [19]. In contrast, our data suggest that these membrane microdomains are

an essential site for PI3K activation during LPA stimulation, implying that lipid rafts also have a positive function in mitogenic signalling. The fact that cholesterol content specifically modulates the PI3K pathway suggests that at least one important component of PI3K signalling is notably compartmentalised in lipid rafts. We thus started to explore the localisation of p85-dependent PI3Ks, taking into account that PI3Kγ is not expressed in Vero cells [7]. Interestingly, p85 was identified in lipid rafts and its localisation was modified by the different MβCD treatments. Because the EGFR is also present in rafts, one may assume that these microdomains represent a privileged location for interaction between EGFR and p85. Moreover, studies addressing the intracellular distribution of the PI3K substrate PtdIns(4,5)P_2 have shown that this lipid is highly concentrated in lipid rafts of different human cells [20,21], although the factors that govern this compartmentalisation remain unknown. Therefore, our results suggest that, besides sequestering EGFR to limit EGF-induced signalling, lipid rafts play a positive role in LPA signalling by bringing together EGFR, PI3K and PtdIns(4,5)P_2, thereby providing a favourable environment for PI3K activation.

Acknowledgements: We thank Claude Vieu and Dr Xavier Collet for cholesterol assay. This work was supported by grants from the Ligue Nationale Contre le Cancer (Comité Tarn-et-Garonne) and from the Association pour la Recherche sur le Cancer, including the ARECA network 'Proteomics and Cancer'.

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