C2C12 Skeletal Muscle Cells Exposure to Phosphatidylcholine Triggers IGF-1 Like-Responses

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Key Words

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Abstract

Glucose uptake by cells in response to stimulation with either IGF-1 or insulin is associated with the translocation of GLUT (glucose transporter) proteins from intracellular cytoplasmic compartments to the plasma membrane. In response to such stimulation, GLUT4 and GLUT1 translocation to the plasma membrane is triggered through an increase in their exocytosis involving phospholipase D (PLD) activation, disrupting the recycling of intracellular GLUT-containing vesicles between the plasma membrane and internal compartments. In skeletal muscle, insulin resistance is observed in association with an increase of dipalmitoyl-phosphatidylcholine, which is also known to interact with PLD. Based on evidence that the recycling process is important for GLUT translocation, we decided to address whether dipalmitoyl-phosphatidylcholine, a non-translocatable phospholipid known to alter the recycling of intracellular vesicles and to interact with PLD, can be

involved in glucose metabolism. We show that an acute change in phospholipid composition, by addition of dipalmitoyl-phosphatidylcholine, leads to GLUT1 translocation to the plasma membrane in conjunction to an increase of Akt and GSK3 β phosphorylation, which are sensitive to PI3K and PLD inhibitors. Moreover, we also show that long-term change in phospholipid composition disrupts both the IGF-1 signalling pathway and GLUT1 partitioning within the cells.

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Introduction

Skeletal muscle accounts for approximately 40% of body mass and for about 70% of glucose disposal [1]. Therefore, Glucose transporter (GLUT) translocation to the plasma membrane plays an important role in insulin like growth factor-1 (IGF-1) or insulin mediated modulation of glucose metabolism, allowing glucose carriage into the cell and its subsequent utilisation or transformation into glycogen [1]. Among these transporters the best

characterised are GLUT1 and GLUT4, with the former being the predominant isoform in myoblasts [1]. Studies in a number of cell types including myoblasts, myotubes and adipocytes, have shown that GLUT4 translocation is dependant on PI3K promoting Akt phosphorylation on its serine residue 473 which in turn deactivates GSK3 β by direct phosphorylation on its serine 9, initiating glycogen synthase activity in concert with GLUT translocation to the plasma membrane [1-3]. GLUT4 has been extensively studied with regard to insulin stimulation of glucose transport in skeletal muscle, however there is little data on the role of GLUT1 in glucose transport in C2C12 myoblasts despite the fact that at this stage of differentiation GLUT1 is expressed at higher levels than GLUT4 [4]. Furthermore, both GLUT4 and GLUT1 have been shown to be translocated in response to insulin in cardiomyocytes and adipocytes [5-7]. Additionally, GLUT1 has also been shown to be involved in glucose transport when translocated to the plasma membrane of C2C12 cells [5]. These observations suggest that GLUT1 plays a major role in glucose transport in C2C12 myoblasts.

Insulin induced GLUT1 or GLUT4 translocation is associated with an increase in their exocytosis [8, 9]. However, the use of K⁺-depleted medium, known to inhibit the clathrin-dependant endocytosis and to alter the fluid phase endocytosis, can also produce an accumulation of glucose transporters at the cell surface, not by increasing their exocytosis but by impairing their endocytosis, suggesting that GLUT transporters continuously recycle between the cell's plasma membrane and internal compartments [10-12]. These observations suggest an intimate relationship between the processes of endocytosis and exocytosis with regard to GLUT redistribution within cells, and suggest that a modulation of either process can play a pivotal role in GLUT translocation by disrupting glucose transporter recycling between internal compartments and the plasma membrane.

A number of studies have shown the importance of plasma membrane phospholipid composition in modulating both endocytosis and exocytosis. It is now well established that the plasma membrane has a non-random phospholipid distribution between its two leaflets. For example, phosphatidylcholine (PC) is essentially maintained in the outer leaflet whereas phosphatidylserine (PS) is preferentially located within the inner leaflet. Such an asymmetrical repartitioning of phospholipids is driven by a putative ATPase-flippase relocating exogenous PS from the outer into the inner leaflet [13-15]. In addition, such a continuous asymmetrical distribution of phospholipids is

also responsible for a higher surface pressure within the inner leaflet triggering the creation of intracellular vesicles [16, 17]. As a consequence, compression of the outer leaflet (e.g. increase in its surface pressure) by addition of phospholipids into the outer leaflet, which are not relocated into the inner leaflet, can lead to equilibration in pressure between the leaflets, inhibiting endocytosis and increasing exocytosis [17, 18].

Interestingly, potential interaction between glucose metabolism and the plasma membrane composition is controversial. However, studies of insulin sensitivity in skeletal muscle have shown that an increase in dipalmitoyl-phosphatidylcholine occurs, both in experimentally induced insulin insensitivity in rats and in type 2 diabetic patients [19-22]. Nonetheless, it is not clear how neutral phospholipids, such as phosphatidylcholine, could impair intracellular glucose metabolism. Recently however, phospholipase D (PLD), known to interact with phosphatidylcholine [23-26], has also been shown to elicit the PI3K/Akt pathway when stimulated with lysophosphatidic acid (LPA) in fibroblasts [27]. Moreover, PLD is known to be activated by both insulin and IGF-1 [28, 29], and has been shown to be involved in the recycling process of intracellular vesicles containing GLUT4 in skeletal muscle [30]. As alteration of the endocytosis process triggers GLUT translocation to the plasma membrane [11], and that such alterations can be obtained by incorporation of non-translocatable phospholipids, such as dipalmitoyl-phosphatidylcholine, into the outer leaflet of the plasma membrane [16, 18], the direct effect of dipalmitoyl-phosphatidylcholine on cellular responses linked to glucose metabolism and GLUT1 translocation of C2C12 myocyte cells were investigated.

Materials and Methods

Cells and materials

C2C12 cells were grown in DME-GlutamaxI containing penicillin (50 μ U/ml) and streptomycin (50 μ g/ml) supplemented with 10% FBS (Gibco BRL-Life Technologies) and maintained at 37°C in 5% CO₂. For all experiments the cells were plated in 12 or 6 well plates pre-coated with collagen Type1 (Sigma). 1-Palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-Glycero-3-Phospho-L-Serine (NBD-PS), 1-Palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-Glycero-3-Phosphocholine (NBD-PC), dipalmitoyl-phosphatidylcholine (PC), dipalmitoyl-phosphatidylserine (PS), palmitoyl-phosphatidylcholine (lysoPC) and palmitoyl-phosphatidylserine (lysoPS) were purchased from Avanti Polar Lipids. FM1 43Fix (referred to in

the text as FM1 43) was purchased from Molecular Probes. Primary antibodies: GLUT1, GLUT4, GSK3 β -p(ser9), Akt1/2/3-p(ser473), p38-p, GSK3 β , Akt, p38, insulin-like growth factor receptor- β (IGFR β) and insulin-like growth factor receptor- α (IGFR α) were purchased from Santa cruz, IGFR β -p was purchased from New England Biolabs. Secondary antibodies: HRP-anti-goat, HRP-anti-rabbit, biotinylated anti-goat and anti-rabbit were purchased from Vector. IGF-1, Tris.HCl, β -mercaptoethanol, bromophenol blue, SDS, glycerol, leupeptin, AEBSF and orthovanadate were purchased from Sigma.

Membrane labelling with NBD-PS and NBD-PC

The method used for NBD-PS or NBD-PC follows previous published protocols [12, 16, 17]. NBD-PC and NBD-PS were dried under nitrogen flow and incubated in serum-free medium at 37°C at a final concentration of 75 μ M and sonicated for 1-minute (Soniprep-150-MSE). 1ml of the solution was then added per well for specific time (as described in the text). After three washes with PBS at 4°C, 500 μ l of PBS-10% BSA (w/v) was incubated at room temperature for 20 seconds. The supernatant containing the fluorescent phospholipids was collected, and 500 μ l of water was added. Phospholipid fluorescence intensity was determined using the Wallac Victor-1420 plate reader (Ex:485nm/Em:535nm). To determine the total fluorescence, following cold washes the cells were directly incubated in 500 μ l water for 30 minutes to lyse cells. The solution was collected and 500 μ l of PBS-10%BSA (w/v) was added and sonicated for 1 minute to allow phospholipid interaction with BSA. Phospholipid fluorescence intensity was then determined using the same technique (plate reader). In each case, we have compared the fluorescence intensity obtained to the fluorescence intensity of 7.5 μ M of the same phospholipid prepared under the same conditions.

LysoPC, lysoPS, PC and PS incubation

Phospholipids in chloroform, prepared at the appropriate concentration (75 nmol/ml/10⁵ cells, 30 nmol/ml/10⁵ cells or 7.5 nmol/ml/10⁵ cells), were dried under nitrogen flow and suspended in DMEM-free at 37°C. After a 1 minute of sonication the solution was immediately added to the cells in culture.

FM1 43 accumulation in cells by endocytosis

The fluorescent probe FM1-43 was used at a final concentration of 5 μ M and incubated with cells 5 minutes prior to fixation with 4% paraformaldehyde (PFA). Confocal microscopy (Zeiss) allowed estimation of relative rates of endocytosis by FM1-43 accumulation in cells as described previously [31].

Immunohistochemistry

The cells were fixed with ice-cold 4% PFA. Following fixation, Saponin-PBS at a concentration of 0.05% (w/v) was added for 1 hour at room temperature to gently permeabilize the plasma membrane. Primary antibody was then incubated at a constant dilution of 1/100 (v/v) during 1hour in Saponin-PBS-BSA containing BSA at 0.5% (w/v). After washing, biotinylated secondary antibody was incubated at a constant

Fig. 1. Differential repartitioning of NBD-PS and NBD-PC within the plasma membrane. NBD-PS and NBD-PC were incubated at 75 nmol/10⁵cells/ml in DMEM. Ratio between total cellular incorporation and plasma membrane outer leaflet localisation of these fluorescent phospholipids were determined by spectrofluorimetry and plotted as a function of time.

dilution of 1/100 (v/v) for 1h at room temperature in Saponin-PBS-BSA. Signal amplification was produced by the use of the peroxidase-tyramide amplification kit (Perkin Elmer Life Sciences) following manufacturer's instructions. Confocal microscopy (Zeiss) and the measurement of fluorescence intensity (EMBL-Zeiss-software) were performed (Objectives used: x50, x60, x70 and x100). To differentiate between GLUT1 located at the plasmalemma from that present at intracellular locations a GLUT1 antibody recognizing only the extra-cellular region of this protein was selected (Santa Cruz). *Western blotting:* To terminate experiments, cells were washed with ice-cold PBS containing orthovanadate (Na₃VO₄) at 0.4 mM and whole cell lysates prepared in lysis buffer (63.5 mM Tris.HCl pH 6.8, 10% glycerol (v/v), 2% SDS (w/v), 1 mM Na₃VO₄, 1mM AEBSF, 50 ig/ml leupeptin and 5% β -mercaptoethanol (v/v)). The protein content of the cell lysate was measured using the Bradford test (Sigma) and a spectrophotometer (spectronic Genesys2), and equal quantities of protein (40 μ g/lane) were

resolved using SDS-PAGE (10% or 7.5%) after addition of 0.02% bromophenol blue (w/v). The gel was then transferred on a Hybond-P membrane (Amersham). Membrane was blocked with non-fat dry milk at 5% (w/v) in PBS-Tween (1/1000 v/v). For immunodetection, primary antibodies were used at a concentration of 1/1000 (v/v) in PBS-Tween for 1 hour; the membrane was subsequently washed 5 times 10 minutes in PBS-Tween. HRP-antibody was added at a concentration of 1/10000 (v/v) in PBS-Tween for 1 hour and the membrane was washed 5 times 10 minutes in PBS-Tween before chemiluminescence reaction was carried out using ECL-plus kit (Amersham). Proteins were visualised using Hyperfilm (Amersham).

Data analysis

For histograms, data are represented by the mean of each experiment $\pm$ standard deviation. Students T-test was performed to determine significance compared to control (*: $P < 0.05$).

Results

PC accumulation in the outer leaflet of the C2C12 plasma membrane

Verification that exogenous phosphatidylcholine (PC) accumulates within the outer leaflet of the plasma membrane of C2C12 cells was carried out by comparing the location of the non-translocatable PC analogue (NBD-PC) within the cell to the translocatable PS analogue (NBD-PS) using the BSA exchange technique. This procedure is widely used to measure the rate of NBD-PS analogue translocation from the outer leaflet into the inner leaflet of cells [16, 17], as well as the measurement of plasma membrane internalisation via fluid phase endocytosis using non-translocatable phospholipid analogues such as NBD-Glucosphingosine [12]. In the present study, C2C12 cells were incubated with 75 μ M of fluorescent phospholipid analogues, namely NBD-PC or NBD-PS, and the amount of each phospholipid in the outer layer of the plasma membrane was measured at given time points. The fluorescence of this fraction was then compared to the total cellular fluorescence. The fluorescence surface/volume ratio for each NBD-phospholipid demonstrates that, after 10 minutes, NBD-PC had accumulated mainly in the outer layer whereas NBD-PS was equally distributed between the inner and the outer leaflet of the plasma membrane (Fig. 1). Moreover, after 90 minutes, 75% of the PC analogue remained within the external leaflet. The exact quantity of PC in the outer leaflet at this time corresponds to 2.5 ± 0.5 nmol/ 10^5 cells (data not shown). This result suggests a flippase-like mechanism involved in PS

Fig. 2. A: Location of GLUT1 transporters after PC or PS incubation. C2C12 cells were pre-incubated in serum-free medium for 3 hours prior treatment without phospholipids (C: control) or with PC, or PS, (at 75 μ M) in serum free for 90 minutes. GLUT1 receptor was subsequently stained both at the plasma membrane and in the cytoplasm. B: Alteration of the endocytosis process upon PC incubation. After 30 minutes of PC treatment, the fluorescent probe FM1 43 was added to a final concentration of 5 μ M for a further 5 minutes preceding cell fixation with cold PFA4%. As described in the text, the total fluorescence visualised is representative of the endocytosis process that is altered upon PC incubation.

analogue translocation from the outer into the inner leaflet, a process that has never previously been demonstrated in myocytes.

GLUT1 translocation to the plasma membrane after PC incubation

To examine the effects of PC incorporation in the plasma membrane on the cellular localisation of GLUT1, we employed immuno-cytochemistry to observe its localisation both in the cytoplasm and at the plasma

Fig. 3. Effect of PC or PS incubation on the phosphorylation of Akt (serine 473) and GSK3 β (serine 9). C2C12 cells were pre-incubated in serum-free medium for 3 hours before PC or PS incubation at 75 μ M for 30 minutes and/or 90 minutes (A, D). C2C12 cells were then labelled to visualize Akt-p and GSK3 β -p, and compared to controls in each case. Confocal pictures show that in the presence of PC, Akt and GSK3 β are phosphorylated within 90 minutes of PC incubation (A). To compare whether or not IGF-1 and PC have an additive effect on the phosphorylation of Akt and GSK3 β , after 30 minutes of PC incubation we added IGF-1 at 250 ng/ml for 1 hour and we compared this result to the incubation of IGF-1 alone at the same concentration also for 1 hour (B). The measurements of the fluorescence intensity of all the experiments are summarized for Akt and GSK3 β respectively (C). To determine whether PS addition has the same effect than PC upon second signalling messengers, we also visualized Akt-p and GSK3 β -p upon PS addition (D).

membrane. To do so, we used a specific antibody recognizing only the extra-cellular region of GLUT1. Therefore, without permeabilizing the plasma membrane, it is possible with this antibody to label GLUT1 at the plasma membrane initially, and after plasma membrane permeabilization to stain GLUT1 within internal compartments secondarily. C2C12 cells were pre-incubated for 3 hours in DMEM-free medium before incubation with PC for 90 minutes. Fig. 2A shows a confocal view of GLUT1 either in the cytoplasm or on the membrane after different treatments. With PC incubation for 90 minutes, a net decrease of cytoplasmic GLUT1 is seen by the drop of fluorescence intensity. In addition, in contrast to the control cells, there is a more

profuse signal at the plasma membrane after PC incubation, demonstrating that exogenous PC accumulation can affect the localisation of GLUT1 within the cells. As exogenous PS is directly relocated from the outer to the inner leaflet, we also examined the effect of PS addition at the same concentration as PC following the same experimental procedure. In contrast to PC, PS incubation does not trigger the externalisation of GLUT1 and remains similar to the control (Fig. 2A). In addition, the fact that PS does not elicit any effect on GLUT1, suggests that the ratio: membrane cholesterol/phospholipids is not involved in PC induced GLUT1 translocation. To determine whether the endocytosis process is altered, after 30 minutes stimulation with PC,

Fig. 4. A: Effect of PC incubation on the phosphorylation of the β subunit of the Insulin (IR) and IGF-1 (IGFR) receptors. C2C12 cells were pre-incubated in serum-free medium for 3 hours before IGF-1 (250ng/ml) or PC (75 μ M) incubation for different times (5, 10, 20 and 30 minutes for IGF-1 and 30, 60 and 90 minutes for PC). Cell lysate was collected to examine the specific phosphorylation of these receptors by western blotting (IR/IGFR β -p). To verify equal loading of proteins the entire β subunit of the IGF-1 receptor (IGFR β tot) was also visualized. **B:** Effect of LY294002 (LY) on PC induces Akt phosphorylation on its serine 473 (Akt-p). 10 minutes before the end

of the 3 hours pre-incubation in serum-free medium, LY was incubated at 10 μ M and maintained at the same concentration over the 90 minutes time period with (LY+PC) or without (LY) PC (75 μ M) incubation. Control and PC incubation only (PC) were also performed.

FM1-43 was subsequently applied. The total accumulation of the fluorescent probe reflecting the rate of endocytosis [31] was compared in C2C12 cells with and without PC incubation (Fig. 2B). As no cell loss occur under these conditions, these data demonstrated that the decrease of fluorescence associated with PC incubation suggests an impairment of the endocytosis process.

Akt and GSK3 β phosphorylation triggered by PC incubation

In response to the observation that PC incubation elicited a change both in the cellular localization of GLUT1 and the endocytosis process, we examined the effects of PC upon Akt and GSK3 β phosphorylation. After PC incubation for 30 or 90 minutes (carried out in the same conditions as above), both Akt-p and GSK3 β -p were labelled using immuno-cytochemistry, in order to measure their fluorescence intensity. Fig. 3A shows the changes in fluorescence within the cells following PC incubation. After 90 minutes of incubation the phosphorylation of Akt and GSK3 β are both elevated (by a factor about 8 to 6, respectively) compared to control cells. Nevertheless, at 30 minutes, GSK3 β -p seems to be more sensitive to PC addition when compared to Akt (Fig. 3C) and we also observed its phosphorylation at a lower PC concentration

(data not shown). Subsequently the effect of PC accumulation in the plasma membrane upon these two second messengers was compared to that produced by IGF-1. To observe whether IGF-1 and PC have additive effect on protein phosphorylation, following an initial pre-incubation of PC for 30 minutes, IGF-1 was subsequently added to the cell (already incubated in the presence of PC) for a further 1-hour period. The results presented in Fig. 3B for Akt-p and GSK3 β -p, demonstrate that in the presence of both PC and IGF-1 the levels of phosphorylated Akt and GSK3 β are higher than those produced by either stimulation (PC or IGF-1) alone. Interestingly, the numerical value of intensities given by the histogram Fig. 3C, shows that the effect of PC and IGF-1 together are additive. To test whether or not PS addition can elicit a similar effect to PC incubation, we incubated C2C12 cells with PS following the same procedure. In contrast to the effect of PC incubation, PS does not trigger either Akt or GSK3 β phosphorylation (Fig.3D).

Akt phosphorylation, upon PC addition, is both IGF-1 and Insulin receptor independent but PI3K dependant

The fact that PC incubation triggers Akt and GSK3 β

Fig. 5. A: Effect of lyso PS (LPS) and lysoPC (LPC) on Akt phosphorylation (Akt-p). Using the same protocol as for PC, LPS or LPC were incubated at a concentration of 7.5 μ M during 30 or 90 minutes. Cell lysate was collected to examine Akt-p. Further to the observation that LPC triggers Akt-p, butanol-1 (But) or LY294002 (LY) were pre-incubated at final concentrations of 1/100 (v/v) or 10 μ M during 1-minute or 10 minutes prior LPC incubation respectively. But and LY were maintained at a concentration of 1/1000 (v/v) or 10 μ M during 90 minutes with (LPC+But, LPC+LY) or without (But, LY and C: control) LPC incubation. B: Effect of butanol-1 (But) on PC induced Akt-p. Pre-incubation of Butanol-1 was done as described above (A) with (PC, PC+But) or without (But and Control) PC incubation at a concentration of 75 μ M. Cells were fixed and Akt-p was visualised.

phosphorylations raises the question as to whether such a phenomenon is linked to the IGF-1 receptor. However no differences in Akt-p levels were observed between cells incubated with 100, 250 or 500 ng/ml of IGF-1 for a 1h treatment (Fig. 9A), suggesting that IGF-1 is used in our experiments at a supra-physiological concentration. These data therefore suggest that 60 minutes of IGF-1 treatment at 250 ng/ml saturates the cell's response to this cytokine and that the excess of Akt and GSK3 β phosphorylations, observed in the presence of both PC and IGF-1 (Fig. 3), is not IGF-1 receptor dependant. To further examine whether insulin or IGF-1 receptors are involved in Akt and GSK3 β phosphorylation upon PC addition, using western blotting we labelled the phosphorylated form of the β subunit of both receptors. To get a positive control, incubation of IGF-1 at 250 ng/ml was performed for different times between 5 and 30 minutes and showed an increased level of the β subunit phosphorylation over this 30 minutes period (Fig. 4A). As PC stimulation elicits Akt phosphorylation at a level similar to IGF-1 at 250 ng/ml, PC incubation was carried

out for different times (30, 60 and 90 minutes). No changes in phosphorylation were detected (Fig. 4A) demonstrating that neither IGF-1 nor insulin receptors (through their β subunit phosphorylation) are responsible for Akt phosphorylation in response to PC incubation.

As Akt phosphorylation is known to be dependent of PI3K activity, the PI3K inhibitor LY294002 was pre-incubated prior to PC addition. The data demonstrates that LY294002 inhibits Akt phosphorylation upon PC incubation, suggesting that PC-dependent Akt phosphorylation involves PI3K activation (Fig. 4B).

Akt phosphorylation is choline head group-dependant

As PC incubation promotes Akt phosphorylation through PI3K, we decided to investigate whether such a response is mainly due to: 1) the aliphatic structure of the phospholipid (e.g. due to two aliphatic chains in the outer leaflet), 2) composition of its polar head (e.g. choline head group dependant), 3) the phospholipid accumulation in the outer leaflet. To differentiate between these possibilities,

Fig. 6. Effects of butanol-1 (But) and LY294002 (LY) on PC induces GLUT1 translocation. C2C12 cells were pre-incubated in serum-free medium for 3 hours before PC (75 μ M) incubation for 90 minutes. When needed, But and LY were pre-incubated at final concentrations of 1/100 (v/v) or 10 μ M during 1-minute or 10 minutes prior PC incubation respectively. But and LY were also maintained at a concentration of 1/1000 (v/v) or 10 μ M with (PC+But, PC+LY) or without (But, LY and Control) during PC incubation (PC). Cells were fixed with PFA4% and GLUT1 was labelled at the plasma membrane. Fluorescence intensity measurements were also performed to determine the level of GLUT1 translocated for each condition.

lysoPC and lysoPS were used both having an identical mono-palmitoyl aliphatic chain. In contrast to PS, lysoPS is not actively relocated into the inner leaflet of the plasma membrane when incubated with cells but maintained, along with lysoPC and PC, in the outer leaflet of the plasma membrane [16, 17]. Therefore, in the first scenario (aliphatic structure in the outer leaflet) only PC should elicit such an effect on PI3K/Akt, in the second case (polar head group dependency) both lysoPC and PC should be involved in this specific stimulation and finally, in the third case (phospholipid accumulation) PI3K/Akt-p activation should be produced by PC, lysoPC and lysoPS.

Lyso-phospholipids were incubated at a concentration to minimize cell detachment from the substrate over 90 minutes, as lyso-phospholipids incorporate more easily into the plasmalemma (data not shown). Following incubation, western blotting was performed to visualise Akt phosphorylation (Fig. 5A). Comparison between lysoPS and lysoPC showed that only lysoPC triggers Akt phosphorylation. The absence of Akt phosphorylation in response to lysoPS is not concentration dependent, as twice the concentration of lysoPS using the same experimental protocol did not lead to elevated Akt-p (data not shown). As no change in Akt phosphorylation was

observed in the presence of PS and lysoPS, this suggests that the mechanism involved in Akt phosphorylation must be choline head group-dependent. Moreover, as LY294002 inhibited PC-induced Akt phosphorylation, we also observed that lysoPC cannot promote Akt phosphorylation in the presence of the PI3K inhibitor. This result demonstrates a similarity between PC and lysoPC with regard to the phosphorylation of Akt (Fig. 5A). Among phospholipases known to interact specifically with PC, PLD (phospholipase D) is known to transform PC into PA (phosphatidic acid) and choline [23-25]. In addition, lysoPC is also known to be a substrate of PLD activity [32]. Therefore, we used butanol-1 known to alter the PLD activity by competing with H₂O in the hydrolysis of the choline head group [23, 33]. We observed that butanol-1 incubation inhibits both lysoPC and PC induced Akt phosphorylation, suggesting the involvement of PLD (Fig. 5A and Fig. 5B).

GLUT1 translocation to the membrane induced by PC is differentially sensitive to LY294002 and butanol-1

As both butanol-1 and LY294002 inhibit PC induced Akt phosphorylation, the effect of these inhibitors on PC

Fig. 7. A: Effect of long-term PC incubation on GLUT1 and GLUT4 expression. PC was incubated overnight at 30 μ M in serum-free medium; cells were subsequently lysed and western blotting was used to observe GLUTs expression with or without treatment. B: Effect of long-term incubation of PC and/or short term incubation of IGF-1 on GLUT1 location at the plasma membrane. C2C12 were incubated overnight with PC at 30 μ M in serum-free medium, the GLUT1 receptors were stained at the plasma membrane (PC), and compared to the location of the GLUT1 receptors for the control incubated in serum-free medium only. To visualise the effect of IGF-1 on GLUT1 receptors, control as well as the cells incubated with PC (PC) were incubated with IGF-1 at 100 ng/ml for 1 hour.

induced GLUT1 translocation were examined. Employing the protocol described above, we determined the level of GLUT1 translocated after 90 minutes of PC incubation by measuring the level of membrane fluorescence intensity (Fig. 6). It was observed that whereas PC in the presence of the PI3K inhibitor LY294002 halves the fluorescence intensity induced by PC alone ($p < 0.05$ for both), butanol-1 strongly decreases the fluorescence intensity to an almost undetectable level that cannot be overcome by PC incubation, suggesting that basal activity of PLD is crucial to maintain GLUT1 transporter at the plasma membrane (Fig. 6).

Effect of Long-term PC incubation on GLUT1 translocation after stimulation by IGF-1

In response to the observation that short term incubation of PC with C2C12 myocytes elicits, at least in part, downstream signalling events similar to those produced by IGF-1 including a change in the location of GLUT1, we examined the affects of long-term incubation with PC upon both the IGF-1 signalling pathway and GLUT1 localisation within cells. A preliminary study was carried out to determine the viability of cells in response to a long-term pre-incubation with PC. At 75 μ M PC overnight, approximately 20% of cells detached but lowering the concentration to 30 μ M reduced this to less than 5% (data not shown). This lower concentration was therefore used for the long-term incubation of PC and immuno-histochemistry carried out for GLUT1. As described above, GLUT1 expression levels were examined using Western analysis and no significant

changes were observed with or without PC pre-incubation overnight (Fig. 7A). To confirm that PC addition overnight does not change the profile of GLUT expression, we carried out a similar experiment for GLUT4. Here again, we did not see any significant change in GLUT4 protein level (Fig. 7A).

As IGF-1 translocates GLUT1 to the plasmalemma, we therefore examined whether this process could be modified by long-term PC incubation. The membrane localisation of GLUT1 was compared with or without IGF-1 exposure for one hour with or without a pre-incubation with PC in serum-free medium overnight (Fig. 7B). Following incubation with IGF-1 alone, we observed the amount of GLUT1 located at the plasma membrane increased. Incubation with PC overnight, produced a similar translocation to the plasma membrane, suggesting that the effect of PC observed during a short-term incubation on GLUT1 location, is still effective after an overnight incubation, even at a lower concentration of PC. Moreover, the effect of PC is similar to that induced by IGF-1. However, PC pre-incubation overnight followed by IGF-1 stimulation for a one-hour period does not show any significant increase in GLUT1 at the plasma membrane, suggesting that the ability of IGF-1 to translocate GLUT1 at the plasma membrane is impaired in this scenario. Thus, as short-term PC incubation triggers, in some respects, the downstream events that are similar to those elicited by IGF-1, it is unsurprising that incorporation of PC overnight might affect the IGF-1 signalling pathway.

Fig. 8. Effect of long-term incubation of PC on the IGF-1 signalling pathway. In order to determine how a long-term incubation of PC at a concentration of 30 μ M in serum-free medium may influence the signalling pathway triggered by IGF-1, the levels of protein phosphorylation for Akt, GSK3 β and p38 after an exposure to IGF-1 at 100 ng/ml for 1 hour were measured. C2C12 cells were pre-incubated overnight in serum-free medium or in serum-free medium plus PC (westerns: lanes C and PC respectively). IGF-1 was then added to C2C12 without or with PC pre-incubation (westerns: Lanes C+ and PC+ respectively). Densitometry measurements show the level of proteins phosphorylation relatively to the control (C) due to IGF-1 pre-incubation (C+) and PC pre-incubation (PC). Changes in protein phosphorylation after PC pre-incubation and addition of IGF-1 (PC+) are represented within the histograms A, B and C.

Effect of Long-term PC incubation on Akt-p, GSK3 β -p and p38-p after IGF-1 stimulation

To assess how the endogenous signalling proteins are affected by an overnight incubation with PC, we focused on three particular proteins involved within the IGF-1 pathway. Akt (Protein Kinase B) is known to be involved in GLUT translocation to the plasma membrane [34]. The kinase GSK3 β activates the glycogen synthase pathway following GLUT translocation to the plasma membrane and is also known to be a target of activated Akt. Finally, as p38 has been shown to be involved in glucose uptake through GLUT activation but not in GLUT translocation to the plasma membrane [35-37] we also decided to examine its level of activation in response to IGF-1. Therefore, C2C12 cells were incubated with or without PC overnight as described above, and then both

were stimulated with IGF-1 at 100 ng/ml for one hour to determine how pre-incubation with PC affects the phosphorylation of these signalling factor by IGF-1. No significant Akt activation, when compared to control levels, after an overnight incubation of PC was observed suggesting that cells are de-sensitized to PC during long-term exposure to this phospholipid (Fig. 8A). In contrast a significant increase of Akt-p was observed in response to IGF-1 in the untreated cells and an even higher increase in phosphorylation in response to this growth factor was measured in cells subjected to PC pre-incubation (Fig. 8A) ($p < 0.05$). However, with GSK3 β phosphorylation, the opposite was observed, such that after PC incubation overnight, IGF-1 has almost no effect on GSK3 β phosphorylation compared to IGF-1 without PC pre-incubation (Fig. 8B) ($p < 0.05$). Nevertheless, we also

observed a higher endogenous effect on GSK3 β -p in response to PC incubation alone (Fig. 8B) ($p < 0.05$). With p38, we observed that IGF-1 did not significantly affect its phosphorylation following pre-incubation with PC. In contrast the effect of IGF-1 without pre-incubation with PC elevated p38 phosphorylation (Fig. 8C) ($p < 0.05$). The observed increase in Akt-p, following PC incubation overnight and subsequent IGF-1 stimulation for one hour might be due to a higher cellular sensitivity to IGF-1. However, incubation of 100 ng/ml of IGF-1 can be considered as already supra-physiological for the C2C12 cells and we confirmed this by demonstrating no difference in Akt-p levels following administration of 100, 250 or 500 ng/ml of IGF-1 for a one hour period (Fig. 9A). Moreover, to examine the effect of PC upon IGF-1R receptor number, the IGF-1R alpha subunit levels were measured but no change was observed (Fig. 9B). This last results suggest that PC incubation overnight contributes to a change in the intracellular signalling process induced by IGF-1 without affecting the affinity of IGF-1 with its receptor.

Discussion

Short-term effects elicited by PC incubation

When the phospholipid dipalmitoyl-phosphatidylcholine (PC) was added to C2C12 myocytes it incorporated predominantly into the outer leaflet, triggering a decrease in endocytosis that preceded an increase in GLUT1 translocation to the plasma membrane as PC induced GLUT1 plasma membrane translocation was altered at 30min following PC incubation (data not shown). Moreover, addition of this phospholipid also triggered Akt and GSK3 β phosphorylation independently of IGF-1 or insulin receptors. Nonetheless, Akt phosphorylation involved PI3K activity, since LY294002 inhibited Akt phosphorylation induced by PC incubation. To further understand the mechanism by which PC addition produces such responses, lysoPS (palmitoyl-phosphatidylserine) and lysoPC (palmitoyl-phosphatidylcholine) were also incubated with the myocytes but only LysoPC was able to elicit Akt phosphorylation. This suggests the involvement of a choline head group-mediated cellular response. To test this, Butanol-1 was used to verify the involvement of PLD with the result that inhibition of Akt was observed in the presence of either PC or lysoPC. Whereas LysoPC is known to be a substrate of PLD, activation of PLD via specific addition of LysoPC to cells has, to our knowledge,

Fig. 9. Effect of different IGF-1 concentration and PC pre-incubation on Akt-p and IGF1R respectively. C2C12 cells were either incubated in DMEM-free medium alone overnight (A) or with addition of PC (30 μ M) (B). A: C2C12 cells were stimulated with different concentration of IGF-1 (100, 250 or 500 ng/ml) for one hour to observe a potential variation in Akt-p level. B: Representative western showing IGF-1 receptor expression by labelling its alpha subunit, with or without PC pre-incubation.

never been reported. Therefore, a possible mechanism of PLD activation could be the transformation of LysoPC to PC that, in turn, activates PLD activity.

Subsequently, PC induced GLUT1 translocation was assessed in the presence of either LY294002 or Butanol-1. Surprisingly, whereas PI3K inhibitor halves the level of GLUT1 translocated to the plasma membrane by PC, butanol-1 dramatically decreases GLUT1 at the plasma membrane to below endogenous levels, which cannot be further increased by PC incubation. This result suggests that basal PLD activity is fundamental both in maintaining GLUT1 at the plasma membrane in non-stimulated C2C12 cells and in triggering GLUT1 translocation in response to PC incubation. As stated in the introduction, the application of the phospholipid PC to C2C12 myocytes was precipitated by two major observations. The first was that PC induces PLD known to be important for GLUT translocation [11]. The second being that addition of non-translocatable exogenous phospholipid (excluding phosphatidylserine or phosphatidylethanolamine) triggers an increase of exocytosis by impairing the native asymmetrical number of phospholipid between the two leaflets of the plasma membrane [16, 18]. As we did not see any change in GLUT1 translocation after LysoPS incubation (data not shown), this suggests that at the

Fig. 10. Pathway involved in PC induced Akt and GSK3 β phospho-rylations involving PLD.

concentration of phospholipid used, the polar head group choline is necessary to trigger the effect we observed both on GLUT1 and the second messengers activation. Therefore, PC incorporation into the plasmalemma can at least in part mimic the intracellular pathway elicited by insulin or IGF-1 and a schematic of this is presented in Fig. 10.

Long-term effects elicited by PC incubation upon IGF-1 stimulation

The effects elicited by PC upon IGF-1 stimulation were also studied over a longer period of time and clear changes were observed in Akt, GSK3 β and p38 phosphorylation, which were independent of the interaction between IGF-1 and its receptor. Interestingly, whereas Akt can be over-phosphorylated after PC incubation overnight in the presence of IGF-1, this was not the case for GSK3 β and p38 phosphorylation. It is noteworthy that studies have shown a clear link between p38 and GSK3 β in myoblast where lithium acts as stimulator of glucose metabolism [38]. Additionally, we have recently shown that p38 phosphorylation can activate GSK3 β -p(S9) in C2C12 cells [39]. Therefore, we may expect that long-term PC incubation could alter this specific axis of glucose metabolism. Moreover, GLUT1 translocation to the plasma membrane increased following long-term PC incubation and cannot be further augmented by IGF-1 stimulation. As no change in GLUT1 protein level is observed after long-term PC incubation, these results suggest that maximal GLUT1 relocation has occurred and that no additional translocation can be elicited by IGF-1. In addition, these data also suggest that Akt phosphorylation is not a major messenger in GLUT1

translocation after overnight treatment with PC, and that accumulation of PC into the plasmalemma may affect the IGF-1 elicited intracellular responses. A possible mechanism by which such long-term effects could be produced might involve breakdown products of PC associated with enhanced PLD activity. However, it has been shown in myoblasts that PC breakdown products generated by PLD activity are used to de novo synthesize PC [40], suggesting that the initial excess of this phospholipid is maintained in the cell. Interestingly, in skeletal muscle from type 2 diabetic subjects, Glycogen Synthase is impaired which has been linked to a lack of GSK3 β inactivation [41-43]. However, even with impaired glycogen synthase, an insulin stimulus to skeletal muscle of type 2 diabetic patients results in a slight increase of Akt phosphorylation on its serine 473, in a manner that resembles the observations in the present study [43].

In conclusion, we support that the differential composition between the two layers of the plasma membrane maybe associated with an impairment of the glucose metabolism, disrupting the Insulin/IGF-1 signalling pathway [21].

Abbreviations

But (Butanol-1); GLUT (Glucose transporter); GSK3 β (Glycogen Synthase Kinase 3 β); IGF-1 (Insulin-like Growth Factor-1); IGFR (insulin-like growth factor receptor); LY (LY294002); LPC (Lyso-phosphatidylcholine); LPS (Lyso-phosphatidylserine); NBDPS (1-Palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-Glycero-3-Phospho-L-Serine);

NBD-PC (1-Palmitoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-*sn*-Glycero-3-Phosphocholine); PC (Phosphatidylcholine), PI3K (Phosphatidylinositol 3 Kinase); PS (Phosphatidylserine).

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