Muscarinic-receptor-mediated inhibition of insulin-like growth factor-1 receptor-stimulated phosphoinositide 3-kinase signalling in 1321N1 astrocytoma cells

Ian H. BATTY¹, Ian N. FLEMING² and C. Peter DOWNES

The Division of Cell Signalling, School of Life Sciences, MSI/WTB Complex, University of Dundee, Dow St, Dundee DD1 5EH, Scotland, U.K.

In 1321N1 astrocytoma cells, stimulation of the IGF-1 (insulin-like growth factor-1) receptor increased the association of PI3K [phosphoinositide (PI) 3-kinase] activity with IRS-1 (insulin receptor substrate 1), and increased the cellular concentration of PtdIns(3,4,5)P₃. Carbachol, acting on M₃ muscarinic receptors, inhibited insulin-, but not PDGF (platelet-derived growth factor)-, stimulated responses by ≈ 50 %. The inhibition of IRS-1-associated PI3K activity by carbachol (i) was rapid (< 1 min), persistent (≥ 60 min) and potent (half-maximal concentration ≈ 1 μM); (ii) was reproduced by stimuli for several phospholipase-C-coupled receptors; (iii) was prevented by the inhibition of protein kinase C, but not by chelation of intracellular Ca²⁺; and (iv) was not blocked or reproduced by inhibitors or stimuli respectively of mitogen-activated protein kinase, PI3K, protein kinase B or the mammalian target of rapamycin. However, the effects of carbachol were prevented by sodium vanadate, a protein tyrosine phosphatase inhibitor, and were accompanied by reduced insulin-stimulated IRS-1 tyrosine phosphorylation and recruitment of the

85 kDa regulatory subunit of PI3K to IRS-1, but not by reduced IGF-1 receptor kinase activity. The inhibitory effect of carbachol was reproduced by okadaic acid, a protein serine/threonine phosphatase inhibitor, but not by PDGF, yet all three agents stimulated the serine phosphorylation of IRS-1 at residues Ser³¹², Ser⁶¹⁶ and Ser^{636/639}, albeit to different extents. Thus muscarinic receptors may inhibit insulin signalling by promoting IRS-1 tyrosine dephosphorylation and/or by uncoupling IRS-1 from the stimulated IGF-1 receptor by stimulating IRS-1 serine phosphorylation. However, the proportion of IRS-1 molecules phosphorylated at a particular site or the phosphorylation of additional IRS-1 serine residues other than those noted above must be important.

Key words: cross-talk, feedback regulation of insulin signalling, insulin receptor substrate, phosphoinositide 3-kinase, phospholipase C.

INTRODUCTION

Phosphoinositides (PIs) are substrates for PLCs (phospholipases C) and PI3Ks (PI 3-kinases), and are targets for protein binding modules with differing PI specificities, such as the pleckstrin and phox homology domains present in many modular proteins [1,2]. PLCs exert their effects by the hydrolysis of PtdIns(4,5)P₂, with the release of Ins(1,4,5)P₃ and *sn*-1,2-diacylglycerol, which, respectively, mobilizes Ca²⁺ from intracellular stores and activates PKC (protein kinase C) [3]. PI3Ks mediate their actions by the synthesis of 3-phosphorylated PIs (3-PIs), which function by their localized recruitment and/or activation of effectors such as PDK1 (phosphoinositide-dependent kinase 1) and PKB (protein kinase B), which express appropriate PI-recognition domains [4,5]. PI3Ks differ in their modes of activation and their preferred PI substrates [5,6]. The type 1, or receptor-activated, PI3Ks use PtdIns(4,5)P₂ as their cellular substrate, converting this into PtdIns(3,4,5)P₃ [7]. The concentration of this intracellular signal is then regulated further by 5- and 3-phosphatase action [8]. The receptor activation of both PLC and PI3K thus places demand on the limited cellular supply of PtdIns(4,5)P₂.

The mechanisms that co-ordinate the use of PtdIns(4,5)P₂ by PLC and PI3K, or integrate the signals which they produce, how-

ever, are poorly understood. To address these issues, we have taken advantage previously of the unique coupling of the insulin or of the IGF-1 (insulin-like growth factor-1) receptor to PI3K to examine the influence which the separate, powerful stimulation of PLC by G_q-coupled receptors exerts on the insulin-stimulated accumulation of 3-PIs [9]. These kinetic studies in 1321N1 astrocytoma cells showed that stimulation of PLC by PARs (protease-activated receptors), i.e. PAR-1, or M₃ muscarinic receptors reduced insulin-stimulated accumulation of PtdIns(3,4,5)P₃. This does not occur, because the availability of PtdIns(4,5)P₂ is limiting, but involves the activation of a PtdIns(3,4,5)P₃ 5-phosphatase and the inhibition of stimulated PI3K. These observations are important, because the recruitment of PI3K and the synthesis of 3-PIs are essential for the control which insulin/IGF-1 exerts over cellular events including glucose transport, protein and glycogen synthesis, and cell survival [10,11]. Defects in these processes or in the activities of the protein and 3-PI phosphatases responsible for terminating these events, therefore, are likely to have profound consequences and may contribute to, for example, the insulin-resistant state that is characteristic of Type II diabetes.

The insulin and related IGF-1 receptors are tetramers composed of two α and β subunits [12]. The α subunits presented at the

Abbreviations used: BAPTA/AM, bis-(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxymethyl ester); ECL, enhanced chemiluminescence; MAPK, mitogen-activated protein kinase; MBP, myelin basic protein; mTOR, mammalian target of rapamycin; PKB, protein kinase B; PKC, protein kinase C; PLC, phospholipase C; IGF-1, insulin-like growth factor-1; IRS, insulin receptor substrate; PAR, protease-activated receptor; PDGF, platelet-derived growth factor; (3-)-PI, (3-phosphorylated) phosphoinositide; PI3K, PI 3-kinase; PKI, inhibitor of protein kinase A; p70S6K, p70 S6 kinase; SH2, Src homology 2; TBS, Tris-buffered saline; TNFα, tumour necrosis factor-α.

¹ To whom correspondence should be addressed (e-mail i.h.batty@dundee.ac.uk).

² Present address: Cyclacel Ltd, James Lindsay Place, Dundee DD1 5JJ, Scotland, U.K.

cell surface constitute the ligand recognition domain which, following occupation, triggers the intrinsic tyrosine kinase activity expressed by the β subunits at the cytoplasmic face of the plasma membrane. The autophosphorylation of the receptor that ensues allows the recruitment and tyrosine phosphorylation by the receptor of targets including the IRS (insulin receptor substrate) proteins [13,14]. The IRS proteins are central to the co-ordination of insulin/IGF-1 signalling due to their capacity to bind key effectors, which express SH2 (Src homology 2) domains such as the adaptor protein Grb2, the protein tyrosine phosphatase SHP-2 and type 1a PI3K. The latter comprises a 110 kDa catalytic subunit and an 85, 55 or 50 kDa regulatory subunit [5,6], the SH2 domains of which mediate its association with tyrosine-phosphorylated IRS-1. Several recent studies have highlighted the importance of serine/threonine phosphorylation of IRS proteins as a means by which both physiological and pathological feedback regulation of PI3K activity is mediated by insulin and other factors [15,16].

Our recent studies in 1321N1 cells have shown that the stimulation of M_3 muscarinic and other receptors which couple via G_q to PLC also activates key mediators of these feedback processes, including PI3K, PKB and mTOR (mammalian target of rapamycin) [17,18]. Here we show that muscarinic receptor stimulation causes inhibition of insulin-stimulated $\text{PtdIns}(3,4,5)P_3$ accumulation, and that this results from the reduced recruitment of PI3K following the enhanced serine/threonine phosphorylation of IRS-1 by novel routes. These results collectively, therefore, reveal that muscarinic receptors exert dual control over PI3K, stimulating activity by promoting the recruitment of PI3K to ErbB3 and inhibiting selectively insulin-stimulated activity via modification of IRS-1. They also indicate that the different agonist potencies with which these opposing actions are regulated allows flexible control of downstream signalling events.

EXPERIMENTAL

Materials

1321N1 astrocytoma cells were from the European Tissue Culture Collection. Antibodies against the following were obtained from the sources indicated: IRS-1, Upstate Biotechnology or Santa Cruz; p85 and anti-phosphotyrosine, Upstate Biotechnology; IGF-1 receptor, Oncogene; IRS-1 anti-phosphoserine antibodies, Cell Signaling Technology or Upstate Biotechnology. Antibodies against the p42 isoform of MAPK (mitogen-activated protein kinase), together with a 20-residue peptide inhibitor of protein kinase A (PKI) [19], were generously provided by the Division of Signal Transduction Therapy, University of Dundee. Raytide[®] was from Oncogene. Reagents for SDS/PAGE and for immunoblotting were obtained from Bio-Rad, Invitrogen and Pierce. Columns for HPLC were obtained from Whatman, and *myo*-[2-³H]inositol (≈ 20 Ci/mmol), [γ -³²P]ATP (5000 Ci/mmol), Protein-G-coupled Sepharose beads and ECL (enhanced chemiluminescence) reagent were from Amersham Biosciences. Other reagents were from CN Biosciences, Alexis Biochemicals, Merck, Sigma or from sources defined previously [9,20].

Cell culture

1321N1 cells were cultured in medium containing or lacking added *myo*-inositol as described previously [20].

Cellular measurements of PLC activity and $\text{PtdIns}(3,4,5)P_3$ accumulation

PLC activity and the cellular accumulation of $\text{PtdIns}(3,4,5)P_3$ were measured using cells cultured in medium containing and

lacking added *myo*-inositol respectively, and in which the inositol phospholipid pools were pre-labelled to steady-state in medium supplemented with *myo*-[2-³H]inositol at 2–5 $\mu\text{Ci/ml}$ and 10–25 $\mu\text{Ci/ml}$ as described previously [9,20].

Preparation of cell extracts

These were prepared as described previously [17].

Immunoprecipitation and *in vitro* assays

Frozen samples of cell lysate were thawed at room temperature (≈ 20 – 25°C) and then centrifuged at 10 000–15 000 g to remove any precipitated material. All enzyme activities were immunoprecipitated for 2 h at 4°C from cell lysates (≈ 1 mg of protein) using antibodies pre-coupled similarly to Protein-G-coupled Sepharose beads (10 μl). All washing and assay buffers were supplemented freshly with sodium vanadate, 1 mM; PMSF, 0.1 mM; benzamidine, 0.1 mM; leupeptin, 20 μM ; and dithiothreitol, 1 mM; or 2-mercaptoethanol, 0.1 % (v/v).

MAPK

MAPK was immunoprecipitated using ≈ 2 μg of antibody. Immunoprecipitated material was washed twice with 1 ml of ice-cold lysis buffer [120 mM NaCl/1 mM MgCl_2 /50 mM NaF/1 mM EDTA/1 mM EGTA/5 mM β -glycerophosphate/1 % (v/v) Triton X-100/25 mM Hepes/NaOH (pH 7.6)] supplemented to 0.5 M with NaCl, and then three times with 1 ml of ice-cold assay buffer [0.1 mM EGTA/0.1 % (v/v) 2-mercaptoethanol/50 mM Tris/HCl (pH 7.5)]. Activity was then assayed using a method based on that described previously [19]. Briefly, washed immunoprecipitates were resuspended in 40 μl of assay buffer pre-warmed to 30°C and supplemented with 0.1 mM ATP, 10 mM MgCl_2 , 0.33 mg/ml MBP (myelin basic protein), 20 μM PKI and 5000 Ci/mmol [γ -³²P]ATP (≈ 1 $\mu\text{Ci/assay}$), and incubated at 30°C for 30 min. Samples of the supernatant (30 μl) were then spotted on to 2 cm^2 sections of p81 phosphocellulose paper, and the papers were washed five times with 0.5–1.0 litre of 75 mM H_3PO_4 and once with ≈ 50 ml of acetone; they were then allowed to dry before quantification of the ³²P product by Cerenkov counting. Sample activity was calculated above a reagent blank comprising the reaction mixture spotted directly on to p81 paper, and processed as above.

IGF-1 receptor kinase

IGF-1 receptor kinase was immunoprecipitated using 1 μg of antibody. The immunoprecipitated material was washed as above, and assayed using a modification of the procedure for MAPK in which Raytide[®] (100 μM) replaced MBP, PKI was omitted and the concentrations of unlabelled and radiolabelled ATP were 1 μM and 25–100 $\mu\text{Ci/assay}$ respectively. Samples were incubated at 30°C , and aliquots (2.5 μl) of the supernatant were collected at 15 min intervals between 0–60 min, spotted on to p81 paper and processed as above. Assays were linear after a short lag period for up to 60 min. No activity was detected in the absence of Raytide[®]. Activity was calculated above a blank as described for MAPK.

PI3K

This was immunoprecipitated using 0.5–1.0 μg (Upstate Biotechnology) or 5 μg (Santa Cruz) of anti-IRS-1 antibody, or with 1.5 μg of anti-phosphotyrosine or 5 μg of anti-p85 antibody. The immunoprecipitated material was then washed and assayed, either as described previously [21] or using a modified procedure.

Briefly, the immunoprecipitated material was washed once with 1 ml of ice-cold lysis buffer and three times with 1 ml of ice-cold assay buffer [1 mM EDTA/1 mM EGTA/2.5 mM MgCl₂/25 mM Hepes/HCl (pH 7.6)], and then resuspended in 40 µl of assay buffer supplemented with unlabelled and radiolabelled ATP at concentrations of 1 µM and 5–10 µCi/assay respectively and phosphatidylinositol/phosphatidylethanolamine vesicles, giving final concentrations of 100 µM with respect to each lipid. The vesicles were prepared as described previously [21]. Samples were then incubated at 37 °C for 30 min, and the reactions were stopped by the addition of 0.6 ml of methanol/chloroform/12 M HCl (80:40:1, by vol.), 0.2 ml of chloroform and 0.32 ml of 0.1 M HCl; the samples were then processed and analysed either by TLC as described previously [21], or in chloroform/methanol/acetone/acetic acid/water (7:5:2:2:2, by vol.), and the spots corresponding to [³²P]PtdIns(3)*P* were identified by autoradiography, excised and quantified by Cerenkov counting. Experiments in which the spots were eluted, deacylated and analysed by HPLC, as described previously [9], confirmed that the lipid product was primarily [³²P]PtdIns(3)*P*.

SDS/PAGE and immunoblotting

Samples of cell lysates (10–20 µg of protein) or of washed immunoprecipitated material were mixed with an equal volume of 2× sample buffer [to give final concentrations of 10 % (v/v) glycerol, 2 % (w/v) SDS, 0.01 % (w/v) Bromophenol Blue and 62.5 mM Tris/HCl, at pH 6.8], and then analysed on either 6 % or 10 % polyacrylamide gels run in Tris/glycine buffer at pH 8.3 [25 mM Tris/HCl/192 mM glycine/0.1 % (w/v) SDS] or on 3–8 % polyacrylamide gels (Invitrogen) developed in Tris/acetate buffers according to the protocol supplied. Protein was then transferred to a PVDF membrane using a semi-dry blotter run at 0.8 mA/cm² for 45 min in Tris buffer [48 mM Tris/HCl (pH 9.5)/39 mM glycine/0.375 % (w/v) SDS/20 % (v/v) methanol]. The membranes were blocked for 1–2 h at room temperature or overnight at 4 °C, either with 5 % (w/v) non-fat milk in TBS (Tris-buffered saline: composition 154 mM NaCl/100 mM Tris/HCl, pH 7.5) or with 5 % (w/v) BSA in TBS if blots were to be probed with anti-phosphotyrosine or anti-phosphoserine antibodies. The membranes were then probed for 2 h at room temperature with antibodies against the indicated target diluted in TBS containing 1 % (w/v) BSA, 0.5 µg/ml IRS-1, 1.0 µg/ml p85 and 0.1 µg/ml anti-phosphotyrosine, or were probed overnight at 4 °C with site-specific anti-phosphoserine antibodies diluted 1:1000. The membranes were then washed three times (10 min each wash) with 50–100 ml of TBS containing 1 % (v/v) Tween 20 (TBST), before incubation for 1 h at room temperature with appropriate goat anti-rabbit or rabbit anti-mouse secondary antibodies (Pierce) diluted to 0.08 µg/ml in TBST. Finally, the membranes were washed as above, but with TBST in place of TBS for the three washes (10 min each wash), followed by one wash in TBS, and then were visualized with ECL reagent and exposed to X-ray film. For some experiments, signals from short-term non-saturated exposures were quantified by densitometry.

RESULTS

Carbachol selectively reduces insulin/IGF-1-stimulated IRS-1-associated PI3K activity and proportionately reduces cellular PtdIns(3,4,5)*P*₃ concentrations

Table 1 shows the results of experiments in which the association of PI3K with IRS-1 in immunoprecipitates of the latter was examined in lysates from cells exposed to maximally effective

Table 1 Carbachol inhibits insulin- and IGF-1-stimulated IRS-1-associated PI3K activity

Cells were incubated for 10 min in the absence (–) or presence (+) of carbachol (1 mM), and with the concentrations of insulin or IGF-1 indicated. The cells were then lysed, IRS-1 was immunoprecipitated and the associated PI3K activity was assayed. The results are expressed as a percentage of the activity measured in response to insulin alone, and represent the mean ± S.E.M. values from three experiments performed in triplicate.

Stimulus	PI3K activity (% of maximum response)	
	– Carbachol	+ Carbachol (1 mM)
None	7 ± 1	7 ± 1
Insulin (100 µM)	100	44 ± 6
IGF-1 (0.1 µM)	98 ± 13	46 ± 4
Insulin (100 µM) + IGF-1 (0.1 µM)	88 ± 7	43 ± 7

concentrations of insulin and/or IGF-1 in the absence or presence of carbachol, a muscarinic receptor agonist which induces persistent stimulation of PLC in 1321N1 cells [20]. Insulin and IGF-1 each stimulated the PI3K activity associated with IRS-1 by approx. 10-fold. The effects of insulin and IGF-1 were not additive, suggesting they are mediated through a common receptor, and since preliminary experiments suggested that IGF-1 acted approx. 10–100-fold more potently than insulin (also compare Table 1 and Figure 1), it is probable that the IGF-1 receptor predominates in these cells. Table 1 also shows that carbachol alone had no effect, but reduced by ≈ 50 % the IRS-1-associated PI3K activity stimulated by insulin and/or IGF-1. This confirms directly our earlier proposal [9] that muscarinic receptor stimuli can inhibit insulin/IGF-1 receptor-stimulated PI3K activity in 1321N1 cells.

Figure 1(A) shows that insulin dose-dependently stimulated IRS-1-associated PI3K activity, with half-maximal (EC₅₀) and maximal effects apparent at 0.3 µM and 10 µM respectively, concentrations which are approx. 3–10-fold higher than those required for activation of PKB in these cells [17]. Figure 1(A) also shows that carbachol acted predominantly to reduce the magnitude, rather than the potency, of the insulin-stimulated response. Consistent with this, Figure 1(B) shows that insulin stimulated the association of the p85 regulatory subunit of PI3K with IRS-1, and that this was reduced similarly by carbachol. This suggests that both the ability of insulin to stimulate, and of carbachol to inhibit, the PI3K activity associated with IRS-1 results primarily from changes in the amount, rather than in the kinetic properties, of the PI3K bound to IRS-1.

Figure 1(C) reveals that changes in the cellular concentrations of PtdIns(3,4,5)*P*₃ in response to insulin alone or in combination with carbachol mirrored those of IRS-1-associated PI3K activity, showing an indistinguishable dose relationship for insulin stimulation, and a proportionately similar reduction in the co-presence of carbachol. This is important for two reasons. First, it shows that the ability of carbachol to reduce insulin-stimulated PtdIns(3,4,5)*P*₃ concentrations reported previously [9] results mainly through effects on the recruitment of PI3K, rather than through an increased capacity to degrade PtdIns(3,4,5)*P*₃. Secondly, it suggests either that IRS-1 is the major IRS protein responsible for the recruitment of PI3K in 1321N1 cells, or that changes at the level of IRS-1 are representative of those across the cellular IRS pool. Preliminary immunoblotting results support the former view, suggesting that while IRS-2 is also present in these cells it is much less readily detected than IRS-1.

In contrast, Table 2 demonstrates that responses stimulated by PDGF (platelet-derived growth factor) were not affected by carbachol. Thus, whereas PDGF stimulated a dramatic increase in

Figure 1 Association of p85 and of PI3K activity with IRS-1, and the increase in cellular PtdIns(3,4,5)P₃ concentration stimulated by insulin are reduced in parallel by carbachol

(A) IRS-1-associated PI3K activity: cells were incubated for 10 min with the concentration of insulin indicated in the absence (closed symbols) or presence (open symbols) of carbachol (1 mM), then the PI3K activity associated with IRS-1 was measured. The results are expressed as a percentage of the increased activity above control values stimulated by insulin alone, and represent the mean values ($\pm$ S.E.M.) from three to seven experiments performed in duplicate. (B) The association of p85 with IRS-1: cells were incubated in duplicate $\pm$ insulin (1 μ M) and $\pm$ carbachol (1 mM) for 10 min and IRS-1, then immunoprecipitated (IP). The immunoprecipitated proteins were analysed by SDS/PAGE on 10% (p85) or 6% (IRS-1) gels and immunoblotted with antibodies for the p85 regulatory subunit of PI3K or for IRS-1. The results are from one experiment (representative of seven) over the total of which the proportionate association of p85 with IRS-1 in control, carbachol- and insulin + carbachol-treated cells respectively was $8 \pm 2\%$, $6 \pm 2\%$ and $54 \pm 8\%$ (means $\pm$ S.E.M.) of that in cells treated with insulin alone. (C) Cellular PtdIns(3,4,5)P₃ concentrations: cells pre-labelled with [³H]inositol were incubated for 10 min with the concentration of insulin indicated in the absence (closed circles) or presence (open circles) of carbachol (1 mM), before the extraction and analysis of the [³H]PIs. The results are expressed as in (A), and represent the mean $\pm$ S.E.M. values for four experiments performed in duplicate.

the cellular concentration of PtdIns(3,4,5)P₃ and in the activity of PI3K associated with anti-phosphotyrosine immunoprecipitates, these were not attenuated by carbachol, although insulin responses in the same experiments were reduced. The selective decrease in insulin, but not PDGF, responses by carbachol suggests an action mediated upstream of PI3K itself. This point is emphasized by the

observation that muscarinic receptor stimulation itself promotes a modest increase in PtdIns(3,4,5)P₃ concentrations in 1321N1 cells [17,18], the scale of which is evident from Table 2. Thus the selective inhibitory effects of carbachol are likely to be mediated through IRS-1 and/or the insulin/IGF-1 receptor.

Muscarinic and other receptors coupled via G_q to PLC induce a potent, rapid and persistent inhibition of insulin signalling

Table 3 shows that stimulation of several G_q-PLC-coupled receptors endogenously expressed in 1321N1 cells, including the M₃ muscarinic [22], PAR-1 [9] and H₁ histamine [23,24] receptors, with maximally effective agonist concentrations all reduced the IRS-1-associated PI3K activity stimulated by insulin. The inhibitory effect of each stimulus was similar, and combinations were not additive, consistent with a common mechanism of action. By contrast, the addition of isoprenaline to stimulate the β -adrenergic receptor, which is coupled to the activation of adenylate cyclase in these cells [25], had no effect.

Figure 2(A) shows that the effects of carbachol were prevented by the muscarinic receptor antagonist atropine, and demonstrates the high potency with which the insulin response is inhibited, with half-maximal (EC₅₀) effects apparent at $\approx 1 \mu$ M carbachol. Because the stimulation of PLC activity by carbachol requires much higher concentrations (EC₅₀ ≈ 20 –40 μ M; [26]), it follows that only a low level of muscarinic receptor occupancy, capable of very limited PLC activation, can be required to inhibit insulin-stimulated PI3K responses. In support of this, Figure 2(A) also shows that arecoline, a potent but partial muscarinic receptor agonist which is approx. 5–10-fold less efficacious than carbachol for the activation of PLC, inhibited insulin-stimulated PI3K activity by ≈ 70 –80% of the extent achieved maximally by carbachol.

Figure 2(B) shows that, in response to insulin alone, PI3K activity associated rapidly with IRS-1 and achieved a new steady-state level within 2–5 min that was largely maintained for at least 1 h. In comparison, in the presence of carbachol the response to insulin was reduced at all times between 30 s and 60 min. Similarly, when cells were first exposed to insulin alone for a period sufficient to allow the association of PI3K with IRS-1 to achieve steady-state, the subsequent addition of carbachol still reduced IRS-1-bound PI3K activity within 1–2 min (results not shown). Figure 2(B) also reveals that, following the addition of an excess of atropine to cells previously exposed to insulin plus carbachol, steady-state levels of IRS-1-associated PI3K returned to those observed in response to insulin alone only over a period of ≈ 10 min. As this is slower than the onset of action of carbachol or insulin, and since signals immediately distal to PLC dissipate very rapidly under these conditions [23,27,28], this recovery period probably reflects that required for reversal of the inhibitory modification of IRS-1 and/or the insulin/IGF-1 receptor induced by carbachol.

Muscarinic-receptor-mediated inhibition of IRS-1-associated PI3K activity requires PKC, but not PI3K, PKB, mTOR/p70S6K (p70 S6 kinase) or MAPK

We examined next the effects of agents which mimic or block signalling downstream of PLC. Table 4 shows that the Ca²⁺ ionophore ionomycin and the phorbol ester PMA, individually or together, reduced insulin-stimulated association of PI3K activity with IRS-1, but to a lesser extent than carbachol. On the other hand, Figure 3 shows that Ro-318220, an inhibitor of PKC, dramatically reduced the potency with which carbachol inhibited the insulin signal, strongly implicating PKC as a mediator of this

Table 2 Carbachol selectively inhibits insulin- but not PDGF-stimulated PI3K

PI3K: Cells were incubated for 10 min as indicated, then lysed and the PI3K activity associated with IRS-1 or anti-phosphotyrosine (P-Tyr) immunoprecipitates was assayed. Results are expressed as a percentage of the activity measured in response to insulin alone or PDGF alone in IRS-1 or anti-phosphotyrosine immunoprecipitates respectively, and represent the mean $\pm$ S.E.M values for three experiments performed in duplicate. Cellular PtdIns(3,4,5) P_3 : cells pre-labelled with [3 H]inositol were incubated for 10 min as indicated, and the [3 H]PIs were then extracted and analysed. The results (d.p.m. $\pm$ S.D.) are from one experiment (representative of three) each performed in triplicate, or show the increase in PtdIns(3,4,5) P_3 in response to insulin or PDGF in the presence of carbachol as a percentage of that in response to each stimulus in the absence of carbachol, and represent the mean $\pm$ S.E.M values for three experiments.

	PI3K (% maximum response)		Cellular PtdIns(3,4,5) P_3	
	IRS-1	P-Tyr	d.p.m. ($\pm$ S.D.)	% Increase $\pm$ carbachol
Stimulus				
Control	9 $\pm$ 1	1 $\pm$ 1	1952 $\pm$ 88	–
Carbachol (1 mM)	8 $\pm$ 1	1 $\pm$ 1	2838 $\pm$ 296	–
Insulin (1 μ M)	100	–	14 440 $\pm$ 1196	–
Insulin (1 μ M) + carbachol (1 mM)	37 $\pm$ 2	–	9147 $\pm$ 400	52 $\pm$ 4
PDGF (50 ng/ml)	–	100	65 867 $\pm$ 5808	–
PDGF (50 ng/ml) + carbachol (1 mM)	–	101 $\pm$ 11	63 102 $\pm$ 5980	98 $\pm$ 2

Table 3 Stimuli for several PLC-coupled receptors inhibit insulin-stimulated PI3K

Cells were incubated for 10 min, as indicated, and the PI3K activity associated with IRS-1 immunoprecipitates was then assayed. The results are expressed as a percentage of the response to insulin alone, and represent the mean $\pm$ S.E.M values for three or four separate experiments, each performed in triplicate. SFLLRN is a hexapeptide agonist for PAR-1.

Stimulus	PI3K (% of maximum response)
Control	11 $\pm$ 4
Insulin (1 μ M)	100
Insulin (1 μ M) + carbachol (1 mM)	48 $\pm$ 6
Insulin (1 μ M) + histamine (1 mM)	61 $\pm$ 4
Insulin (1 μ M) + SFLLRN (100 μ M)	54 $\pm$ 5
Insulin (1 μ M) + isoprenaline (100 μ M)	100 $\pm$ 4
Insulin (1 μ M) + carbachol (1 mM) + histamine (1 mM)	50 $\pm$ 5
Insulin (1 μ M) + carbachol (1 mM) + SFLLRN (100 μ M)	52 $\pm$ 9

response. However, as Ro-318220 is known to interact with targets other than PKC [29] and may even act as a weak muscarinic receptor antagonist [30], we sought to confirm this conclusion by additional means. Table 5 therefore examines the effects of saturating concentrations of the partial muscarinic receptor agonist, arecoline, in untreated cells and those in which PKC was either inhibited by Ro-318220 or down-regulated by chronic phorbol ester treatment [31,32]. In the absence of inhibitors, arecoline reduced the insulin response by $\approx$ 50%. In cells exposed to Ro-318220 or chronic PMA respectively, the inhibitory effect of arecoline was substantially reduced, or was abolished. This combination of approaches therefore clearly identifies a phorbol-ester-sensitive PKC as an important component of the inhibitory mechanism activated by muscarinic receptors. Interestingly, Table 5 also shows that both Ro-318220 and chronic PMA treatment enhanced the association of PI3K activity with IRS-1 stimulated by insulin alone, implying additional roles for PKC in the feedback regulation of insulin signalling.

In addition, Table 5 reveals the effects of pre-loading cells with the cell-permeant Ca^{2+} chelator BAPTA/AM [bis-(*o*-amino-phenoxy)ethane-*N,N,N',N'*-tetra-acetic acid tetrakis(acetoxy-methyl ester)]. This shows that the dampening of muscarinic-receptor-mediated Ca^{2+} signals did not block the inhibitory action of arecoline. This is important, because the Ca^{2+} -dependent activations of mTOR/p70S6K and of PI3K and PKB in response to muscarinic receptor stimulation in 1321N1 cells are both blocked

by BAPTA/AM [17,18]. Thus none of these is likely to mediate the inhibitory actions of muscarinic receptor stimulation. Three further observations support this conclusion. First, Table 4 shows that PDGF, which powerfully activates PI3K in 1321N1 cells (see Table 2) but couples to PLC only very weakly with a response that desensitizes within 15 min (I. H. Batty and C. P. Downes, unpublished work), had no effect on insulin-stimulated recruitment of PI3K activity to IRS-1. Secondly, rapamycin (100 nM), a potent inhibitor of mTOR that completely prevents muscarinic receptor activation of p70S6K [18], did not reduce the ability of carbachol to inhibit the insulin response (results not shown). Thirdly, LY294002 (50 μ M), a reversible inhibitor of PI3K that also inhibits PI 4-kinase and limits PLC activity [33] and blocks mTOR [34], reduced the effects of carbachol only to a limited extent, compatible with its action on PI 4-kinase/PLC activity (results not shown).

The role of MAPK in mediating the current inhibitory effects was also examined using the potent MAPK kinase inhibitor U0126. Figure 4(A) shows that both muscarinic and insulin receptor stimulation, individually or in combination, activated MAPK by approx. 2–4-fold, and that U0126 powerfully inhibited these responses. Figure 4(B) shows that, despite its effectiveness in blocking MAPK activity, U0126 did not prevent carbachol inhibition of insulin-stimulated PI3K activity within the same cell extracts. Thus we can be confident that, whereas PKC is important, MAPK, PI3K, PKB, mTOR and p70S6K do not mediate the inhibitory effects of carbachol on insulin-stimulated responses.

Carbachol inhibition of insulin signalling is accompanied by reduced IRS-1 tyrosine phosphorylation that is not the result of reduced IGF-1 receptor function

As IRS-1 and/or the insulin/IGF-1 receptor are probable targets through which the inhibitory effects of muscarinic receptor stimulation are mediated, plausible mechanisms may involve reduced IRS-1 tyrosine phosphorylation and/or impaired receptor kinase function. Figure 5(A) explores the role of protein tyrosine phosphatase activity in the inhibitory mechanism by examining the effects of sodium vanadate. This shows that, in addition to dramatically increasing basal and insulin-stimulated IRS-1-associated PI3K activity, consistent with its established insulin-mimetic effects [35,36], sodium vanadate prevented the inhibition of the insulin response by carbachol. This suggests that functional protein tyrosine phosphatase activity is essential to the inhibitory

Figure 2 The concentration-dependence and time course for carbachol inhibition of insulin-stimulated PI3K

Concentration-dependence: cells were incubated for 15 min in the absence or presence of atropine as indicated, then incubated further for 10 min in the absence or presence of insulin alone or in combination with carbachol or arecoline at the indicated concentrations, and the PI3K activity associated with IRS-1 was then measured. The results are expressed as a percentage of the response to insulin alone and represent the means $\pm$ S.E.M. of 5–27 experiments performed in duplicate. Control levels of PI3K activity associated with IRS-1 were not altered by carbachol, arecoline or atropine alone. (B) Time course: cells were incubated for the times indicated with vehicle (squares) or with insulin (1 μ M) alone (closed circles) or together with carbachol (1 mM) (open circles), or were first incubated for 5 min with insulin (1 μ M) plus carbachol (50 μ M) and then atropine (10 μ M) was added (triangles), and incubations were allowed to continue for a further 2, 5 or 10 min. The PI3K activity associated with IRS-1 was then measured. The results are expressed as a percentage of the maximum response to insulin alone, which was achieved after 5 min, and represent the mean $\pm$ S.E.M. values for three or four experiments performed in duplicate or triplicate.

Table 4 PMA and ionomycin, but not PDGF, inhibit insulin-stimulated PI3K

Cells were incubated for 10 min as indicated, and the PI3K activity associated with IRS-1 was then measured. The results are expressed as a percentage of the response to insulin alone, and represent the mean $\pm$ S.E.M. values for 6–12 experiments each performed in duplicate or quadruplicate.

Stimulus	PI3K (% of maximum response)
Insulin (1 μ M)	100
Insulin (1 μ M) + carbachol (1 mM)	45 $\pm$ 2
Insulin (1 μ M) + PMA (1 μ M)	74 $\pm$ 6
Insulin (1 μ M) + ionomycin (1 μ M)	78 $\pm$ 6
Insulin (1 μ M) + ionomycin (1 μ M) + PMA (1 μ M)	67 $\pm$ 6
Insulin (1 μ M) + PDGF (50 ng/ml)	104 $\pm$ 10

Figure 3 Muscarinic-receptor-mediated reduction of insulin-stimulated PI3K activity is blocked by inhibition of PKC activity

Cells were incubated for 15 min with DMSO (0.1 %, v/v) vehicle (circles) or with Ro-318220 (10 μ M) (triangles), before incubation for a further 10 min with insulin (1 μ M) plus carbachol at the concentration indicated, and the PI3K activity associated with IRS-1 was then measured. The results are expressed as a percentage of the maximum response to insulin in the absence or presence of Ro-318220 respectively, and represent the mean $\pm$ S.E.M. values for seven to nine experiments performed in duplicate.

Table 5 The muscarinic-receptor-mediated inhibition of insulin-stimulated PI3K requires PKC activity, but not increased Ca^{2+} concentrations

Cells were pre-incubated for 30 min with DMSO (0.1 %, v/v) vehicle or with Ro-318220 (10 μ M) in incubation buffer, or were pre-loaded with BAPTA/AM (40 μ M) or DMSO vehicle (1.0 %, v/v) for 60 min in tissue-culture medium lacking serum, and then washed with incubation buffer and pre-incubated for 30 min. For PKC down-regulation, cells were treated overnight with PMA (1 μ M) or DMSO vehicle (0.25 %, v/v) in complete culture medium, then washed with incubation buffer and pre-incubated for 30 min. All cells were then incubated further for 10 min $\pm$ insulin (1 μ M) and $\pm$ arecoline (1 mM), and the PI3K activity associated with IRS-1 was then assayed. The results are expressed as a percentage of the response to insulin alone, and represent the mean $\pm$ S.E.M. values for five experiments, each performed in duplicate. Values for samples incubated without insulin are omitted for simplicity and were not affected by Ro-318220 or chronic PMA, but were reduced by $\approx$ 60 % by BAPTA/AM.

Inhibitor	PI3K ...			
	– Inhibitor		+ Inhibitor	
	Insulin	Insulin + arecoline	Insulin	Insulin + arecoline
Ro-318220	100	46 $\pm$ 1	175 $\pm$ 22	148 $\pm$ 10
BAPTA/AM	100	57 $\pm$ 6	76 $\pm$ 4	46 $\pm$ 2
PKC down-regulation	100	50 $\pm$ 3	182 $\pm$ 31	202 $\pm$ 22

mechanism. In support of this, Figure 5(B) reveals that carbachol, although without effect in the absence of insulin, reduced in parallel the insulin-stimulated tyrosine phosphorylation of IRS-1 and the stimulated association with IRS-1 of p85 and of PI3K activity, but did not alter the tyrosine phosphorylation of the IGF-1 receptor. The last point is demonstrated quantitatively by Figure 5(C), which shows that the marked IGF-1 receptor kinase activity stimulated by insulin was not reduced by carbachol, which nevertheless effectively inhibited insulin-stimulated PI3K activity in the same cell extracts. These results show that a reduction in the insulin-stimulated tyrosine phosphorylation of IRS-1 is essential to the inhibitory mechanism activated by muscarinic receptors, but is achieved independently of attenuated IGF-1 receptor function.

Figure 4 Muscarinic-receptor-mediated inhibition of insulin-stimulated PI3K activity is not exerted via MAPK

Cells were pre-incubated for 30 min with DMSO (0.1% v/v) vehicle or with U0126 (10 μ M), then incubated further for 10 min $\pm$ insulin (1 μ M) and $\pm$ carbachol (1 mM), before the MAPK activity (A) or IRS-1-associated PI3K activity (B) was immunoprecipitated from common lysates and assayed. The results are expressed as a percentage of control values (MAPK) or of the response to insulin alone (PI3K), and represent the mean $\pm$ S.E.M. values for four experiments performed in triplicate.

Muscarinic-receptor-mediated inhibition of insulin signalling is reproduced by okadaic acid, and accompanied by serine phosphorylation of IRS-1

Recently, a number of receptor and other stimuli have been shown to promote the serine phosphorylation of IRS-1, functionally uncoupling it from the insulin/IGF-1 receptor and/or its downstream effectors [37–43]. The results presented in Figure 6 suggest that similar mechanisms can be activated in 1321N1 cells. Figure 6(A) shows that okadaic acid, an inhibitor of the protein serine phosphatases 1 and 2A (PP1 and PP2A) [44], dose-dependently reduced insulin-stimulated IRS-1-associated PI3K activity, with half-maximal effects at ≈ 0.3 μ M, which were of a similar magnitude to those achieved by 1 mM carbachol. Higher concentrations of okadaic acid almost completely prevented the insulin response. Figure 6(C) also shows that okadaic acid correspondingly reduced insulin-stimulated increases in the cellular concentration of

Figure 5 Roles of tyrosine phosphatase and IGF-1 receptor tyrosine kinase activities in the inhibition of insulin-stimulated PI3K by carbachol

(A) The effects of sodium vanadate on IRS-1-associated PI3K activity: cells were pre-incubated for 30 min in the absence or presence of sodium vanadate, then incubated for 10 min with vehicle, insulin (1 μ M) or insulin (1 μ M) plus carbachol (1 mM) as indicated, and the PI3K activity associated with IRS-1 was then measured. The results are expressed as a percentage of the response to insulin alone, and represent the mean $\pm$ S.E.M. values for four experiments performed in triplicate. (B) The effects of carbachol on insulin-stimulated activation of IRS-1 and of the IGF-1 receptor: cells were incubated $\pm$ insulin (1 μ M) and $\pm$ carbachol (1 mM) for 10 min. Lysates were then prepared and pooled from six separate incubations, and duplicate aliquots were taken to allow: (i) immunoprecipitation of IRS-1 and assay of the associated PI3K activity, as indicated by the values shown which represent the means $\pm$ S.E.M. for four experiments; (ii) immunoprecipitation of IRS-1 and the SDS/PAGE analysis of the immunoprecipitated proteins, followed by immunoblotting with antibodies for p85, phosphotyrosine or IRS-1; or (iii) immunoprecipitation (IP) of the IGF-1 receptor followed by SDS/PAGE analysis and immunoblotting with antibodies against phosphotyrosine. Immunoblots are from a single experiment (representative of four) which gave similar results, and over the total of which the proportionate association of p85 with IRS-1 or tyrosine phosphorylation of IRS-1 in control, carbachol- and insulin + carbachol-treated cells was $8 \pm 3\%$, $8 \pm 4\%$ and $68 \pm 5\%$, or $26 \pm 13\%$, $27 \pm 9\%$ and $62 \pm 13\%$ (means $\pm$ S.E.M.) respectively of that in cells treated with insulin alone. The tyrosine phosphorylation of the IGF-1 receptor in control, carbachol and insulin + carbachol-treated cells over the same experiments was $1 \pm 1\%$, $1 \pm 1\%$ and $84 \pm 10\%$ (means $\pm$ S.E.M.) respectively of that in cells treated with insulin alone. (C) Carbachol inhibits insulin-stimulated PI3K, but not IGF-1 receptor kinase activity: cells were incubated for 10 min $\pm$ insulin (1 μ M) and $\pm$ carbachol (1 mM), then measurements were made of the PI3K activity or the tyrosine kinase activity associated respectively with immunoprecipitates of IRS-1 or of the IGF-1 receptor made from common lysates. The results are expressed as a percentage of the activity measured in response to insulin alone, and represent the means $\pm$ S.E.M. for four experiments.

PtdIns(3,4,5) P_3 . In comparison, okadaic acid had a much lower effect on PDGF-stimulated PtdIns(3,4,5) P_3 accumulation and, as shown in Figure 6(B), had no direct effect on PI3K itself,

Figure 6 The muscarinic-receptor-mediated inhibition of insulin-stimulated PI3K is reproduced by okadaic acid

(A) and (B) PI3K: cells were pre-incubated for 30 min in the absence or presence of okadaic acid (OA) at the concentrations shown, before treatment for 10 min $\pm$ insulin and $\pm$ carbachol as indicated, and the PI3K activity associated with (A) IRS-1 or (B) the p85 regulatory subunit of PI3K was then assayed. The results are expressed as a percentage of the response to insulin alone, and represent the mean $\pm$ S.E.M. values for three experiments performed in triplicate. (C) Cellular PtdIns(3,4,5)P₃: cells pre-labelled with [³H]inositol were incubated for 30 min $\pm$ okadaic acid (OA; 1 μ M) before treatment for 10 min $\pm$ insulin or PDGF, as indicated, and the [³H]phosphoinositides were then extracted and analysed. The results represent the mean $\pm$ S.E.M. values for three experiments performed in duplicate, and are expressed as a percentage of the response to insulin alone.

since the activity of this enzyme in p85 immunoprecipitates was not reduced. These results are consistent with an earlier report that okadaic acid promotes serine phosphorylation of IRS-1 and diminishes its capacity to recruit PI3K [45], and establish that an

agent which acts upstream of PI3K in this manner can reproduce the inhibitory effects of carbachol.

The phosphorylation of IRS-1 at Ser³⁰⁷ and Ser⁶¹² (Ser³¹² and Ser⁶¹⁶ in human IRS-1), and at Ser^{636/639} has been implicated in the feedback control of insulin signalling [37–40,42,43]. Figure 7 shows the results of experiments in which site-specific anti-phosphoserine antibodies were used to examine the phosphorylation of IRS-1 at these residues. The results show that, while insulin stimulated modest phosphorylation of Ser⁶¹⁶, Ser^{636/639} and Ser³¹², carbachol and arecoline dramatically stimulated phosphorylation of IRS-1 at Ser⁶¹⁶, Ser^{636/639} and, to a much lesser extent, Ser³¹², irrespective of the presence of insulin. Higher concentrations of okadaic acid (1.0 μ M) stimulated greater phosphorylation of all three sites, accompanied by a reduced mobility of IRS-1 on SDS/PAGE. The effects of PDGF and of lower okadaic acid concentrations are of particular interest since, regardless of the presence of insulin, PDGF-stimulated phosphorylation of all three sites was at least equal to that evoked by 0.3 μ M okadaic acid, and yet only the latter inhibited insulin-stimulated PI3K responses. The implications of these results are discussed below.

DISCUSSION

Our results show that insulin or IGF-1, acting predominantly via an IGF-1 receptor, stimulates the association of PI3K with IRS-1, and that muscarinic receptor activation reduces this response. This inhibitory effect is accompanied by the decreased recruitment of the p85 regulatory subunit of PI3K to IRS-1, and an equivalent reduction in the cellular accumulation of PtdIns(3,4,5)P₃, the primary lipid mediator of PI3K actions. The inhibitory effect on PI3K activity is reproduced by stimuli for other receptors that couple to PLC, is exerted over a time-scale compatible with PLC activation and is blocked by pharmacological tools which inhibit PKC, but not Ca²⁺ signalling downstream of PLC activity. The time-course data are of particular interest, because they show that insulin-stimulated association of PI3K with IRS-1 achieves a steady-state within 1–2 min and that a secondary, lower steady-state is reached within a similar time following the addition of carbachol to insulin-stimulated cells. This suggests that the half-time of the IRS-1–PI3K complex is less than this interval and, since the time course for insulin-stimulated accumulation of PtdIns(3,4,5)P₃ mirrors that for IRS-1-associated PI3K activity (compare Figure 2B and results in [9]), its half-life must also be of a similar order or less. The maintenance of an active IRS-1–PI3K complex capable of sustained PtdIns(3,4,5)P₃ production must therefore require continual re-phosphorylation by the insulin/IGF-1 receptor. As IRS-1 may exert some of its actions at sites remote from the plasma membrane [46], this suggests either that dynamic mechanisms exist for its recycling to the receptor and/or that an activated fraction is less vulnerable to tyrosine phosphatase activity.

Our results also reveal that the selective inhibitory actions of PLC stimuli on insulin/IGF-1 responses are achieved via IRS-1, which shows a comparable reduction in tyrosine phosphorylation and associated PI3K activity. This occurs without concomitant impairment of IGF-1 receptor tyrosine kinase activity, but the reduction in insulin-stimulated IRS-1-associated PI3K activity mediated by muscarinic receptors is prevented by the inhibition of tyrosine phosphatase activity. This is important, because it implies that the mechanism leading to the reduced association of PI3K with tyrosine-phosphorylated IRS-1 does not involve modifications that decrease the affinity of either component for the other independently of tyrosine phosphatase activity. It follows that PLC stimuli may act either by increasing tyrosine phosphatase activity directed against IRS-1, or by modifying IRS-1 so that it

Figure 7 Serine phosphorylation of IRS-1 is stimulated by carbachol

Cells were pre-incubated for 30 min $\pm$ okadaic acid at the concentrations indicated, then incubated further for 10 min with vehicle or with carbachol, arecoline or PDGF, each in the absence or presence of insulin (1 μ M). Samples of the cell lysates obtained were then either analysed by SDS/PAGE and the resolved proteins immunoblotted with antibodies for IRS-1 and for the indicated phosphoserine residues of IRS-1, or lysates were immunoprecipitated with antibodies for IRS-1 and the activity of the associated PI3K was assayed. Immunoblots are from one experiment (representative of three), which gave very similar results. PI3K activity values represent the means $\pm$ S.E.M. for the same three experiments.

is less efficiently phosphorylated by the active IGF-1 receptor kinase, and its steady-state level of tyrosine phosphorylation declines as a result of constitutive phosphatase activity.

A recent study [47] has proposed that G_q -coupled receptors may mediate an increase in protein tyrosine phosphatase activity, allowing a general inhibitory effect to be exerted on the activation of PI3K by growth-factor receptors. However, this is clearly contrary to the selective effects on insulin/IGF-1-stimulated responses reported here. Moreover, the effects of okadaic acid and the direct demonstration that muscarinic receptor stimulation evokes serine phosphorylation of IRS-1 both suggest a mechanism in which this results in a partial uncoupling of IRS-1 from the IGF-1 receptor.

Recently, a number of serine/threonine kinases, including PI3K, PKB, mTOR, PKC and MAPK [15,16], have been shown to phosphorylate IRS-1 with both positive and negative functional consequences. Typically, negative feedback regulation of IRS-1 mediated acutely by serine/threonine phosphorylation of IRS-1 occurs by uncoupling of IRS-1 from either its upstream receptor or its downstream effectors, and mechanisms of this type are triggered by diverse stimuli. For example, the activation of MAPK by endothelin or phorbol esters causes phosphorylation of IRS-1 at Ser⁶¹² (Ser⁶¹⁶ in humans), inhibiting its insulin-stimulated association with PI3K [42,43]. Similar consequences for insulin signalling in response to PDGF or TNF α (tumour necrosis factor- α) respectively have been reported to involve PI3K, PKB or mTOR-dependent phosphorylation of IRS-1 at Ser⁶³², Ser⁶³⁶ and Ser⁷³¹ (in humans, Ser⁶³⁶, Ser⁶³⁹, Ser⁷³⁶) [42,43] or Ser^{636/639} [40]. Additionally, TNF α and other stimuli acting via MAPK or stress-activated protein kinase phosphorylate IRS-1 at Ser³⁰⁷ (human, Ser³¹²), resulting in its uncoupling from the insulin receptor [37–39].

In 1321N1 cells, muscarinic [48] and PAR-1 [49] receptor stimuli activate MAPK, and receptors coupled via G_q to PLC mediate a Ca^{2+} -dependent transactivation of ErbB3, leading to the recruitment of PI3K and the activation of PKB [17]. Muscarinic receptors also mediate a separate Ca^{2+} -dependent and powerful stimulation of mTOR and p70S6K activities [18]. The activation of these pathways is thus consistent with the ability of muscarinic receptor stimuli to induce phosphorylation of IRS-1 at Ser⁶¹⁶, Ser^{636/639} and Ser³¹². However, it is clear that the inhibition of insulin-stimulated responses exerted by muscarinic receptors is not mediated by MAPK, PI3K, PKB or mTOR/p70S6K, since this is neither prevented by inhibitors of these activities, nor reproduced by their stimulation by PDGF. This suggests either that muscarinic receptor stimuli may induce serine phosphorylation of IRS-1 by alternative routes, or that their inhibitory effect on insulin signalling relies on the phosphorylation of IRS-1 at additional residues and/or is achieved by separate means. A requirement for the phosphorylation of additional residues is the simplest explanation, and is compatible with the effects of PDGF and okadaic acid. Thus PDGF and low concentrations of okadaic acid both stimulated phosphorylation of IRS-1 at Ser⁶¹⁶, Ser^{636/639} and Ser³¹², albeit to a lesser extent than did carbachol, but only okadaic acid inhibited insulin-stimulated PI3K activity. This implies that okadaic acid exerts its inhibitory effects via the phosphorylation of IRS-1 at additional residues, as suggested previously [45]. It also suggests that the ability of muscarinic stimuli to inhibit insulin signalling may rely both on the phosphorylation of similar additional sites and on the fraction of IRS-1 molecules that are phosphorylated at specific sites.

A variety of PKC isoforms have been implicated in the regulation of insulin signalling, mediating effects on both the insulin receptor and IRS proteins (see [13,15] and references

therein). In SH-SY5Y neuroblastoma cells, muscarinic-receptor-mediated inhibition of insulin-stimulated PI3K correlates with the accumulation of CMP-phosphatidate [50], a lipid produced when *sn*-1,2-diacylglycerol is recycled to allow the synthesis of new phosphatidylinositol following the stimulation of PLC. This is consistent with our view that the inhibitory effects of PLC stimuli are mediated via PKC. In 1321N1 cells, however, the roles that we have identified for PKC are novel in several regards. Notably, PKC is involved in the regulation of insulin-stimulated PI3K activity at two levels; first, mediating the inhibitory effects of muscarinic receptor stimulation and secondly, mediating either a negative feedback control activated by insulin itself or a tonic suppression of either insulin-stimulated or PI3K responses in general. The first is shown clearly by its susceptibility to prevention by PKC blockade or down-regulation and, most probably, reflects a role for PKC α or ϵ , since, of the α , δ , ϵ and ζ isoforms expressed in 1321N1 cells [32,51,52], these are most comprehensively down-regulated by chronic PMA treatment [32]. Furthermore, our results suggest that novel signalling components may be involved downstream of this PKC, because muscarinic-receptor-mediated inhibition of insulin-stimulated PI3K is not sensitive to the inhibition of MAPK that mediates the otherwise very similar effects of PMA and endothelin reported previously [42,43]. This is consistent with our view that the muscarinic-receptor-mediated effects involve phosphorylation of IRS-1 at a distinctive set of serine residues. The second role for PKC is revealed by the increased PI3K activity observed in response to insulin alone following PKC inhibition or down-regulation. This may involve actions exerted by PKC ζ , which has been shown to be involved in the feedback control of insulin signalling [53,54] and is both expressed and partially down-regulated by chronic PMA treatment in 1321N1 cells [32].

The influence which the inhibitory effect of PLC activation on insulin-stimulated responses exerts on downstream events dependent on PI3K activity is likely to be complex. In addition to inhibiting potently insulin-stimulated PI3K activity, muscarinic receptor agonists also act approx. 10–30-fold less potently to stimulate independently PKB, mTOR and p70S6K activities [17,18]. These effects are all mediated via the stimulation of PLC, the magnitude and duration of action of which varies greatly in response to agonists for different G_q -coupled receptors. Thus the net effect of co-stimulation with ligands for these receptors and with insulin/IGF-1 will vary predictably, depending on the concentration and duration of each stimulus and on the downstream response measured. At low doses, for example, muscarinic receptor agonists would be expected to exert a persistent and, predominantly, inhibitory action on insulin-stimulated responses that would be offset by independent stimulatory effects at higher concentrations.

In conclusion, we have demonstrated that muscarinic and other receptors coupled via G_q to PLC mediate a potent inhibition of insulin/IGF-1-stimulated PI3K activity, which accounts largely for their ability to lower stimulated cellular concentrations of $\text{PtdIns}(3,4,5)\text{P}_3$. This action is mediated via PLC and PKC, and is accompanied by increased serine phosphorylation of IRS-1, although similar phosphorylation of additional residues and/or other events may be involved in the inhibitory effects on PI3K activity. The future clarification of these issues should provide valuable insight into the feedback regulation of insulin signalling and the integrated control of cellular events by PLC and PI3K.

Financial support from Medical Research Council, U.K. (Programme Grant No. G9403619) is gratefully acknowledged. We are grateful also to Dr Simon Clough for his contribution to these studies, and to Mrs J. Batty for her careful assistance in the preparation of this manuscript.

REFERENCES

- Pawson, T. and Nash, P. (2003) Assembly of cell regulatory systems through protein interaction domains. *Science* **300**, 445–452
- Lemmon, M. A. (2003) Phosphoinositide recognition domains. *Traffic* **4**, 201–213
- Berridge, M. J. (1993) Inositol trisphosphate and calcium signalling. *Nature (London)* **361**, 315–325
- Vanhaesebroeck, B. and Alessi, D. R. (2000) The PI3K–PDK1 connection: more than just a road to PKB. *Biochem. J.* **346**, 561–576
- Vanhaesebroeck, B., Leeyers, S. J., Ahmadi, K., Timms, J., Katso, R., Driscoll, P. C., Woscholski, R., Parker, P. J. and Waterfield, M. D. (2001) Synthesis and function of 3-phosphorylated inositol lipids. *Annu. Rev. Biochem.* **70**, 535–602
- Wymann, M. P., Zvelebil, M. and Laffargue, M. (2003) Phosphoinositide 3-kinase signalling – which way to target? *Trends Pharmacol. Sci.* **24**, 366–376
- Stephens, L. R., Jackson, T. R. and Hawkins, P. T. (1993) Agonist-stimulated synthesis of phosphatidylinositol(3,4,5)-trisphosphate: a new intracellular signalling system? *Biochim. Biophys. Acta* **1179**, 27–75
- Leslie, N. R. and Downes, C. P. (2002) PTEN: the down side of PI 3-kinase signalling. *Cell Signal.* **14**, 285–295
- Batty, I. H. and Downes, C. P. (1996) Thrombin receptors modulate insulin-stimulated phosphatidylinositol 3,4,5-trisphosphate accumulation in 1321N1 astrocytoma cells. *Biochem. J.* **317**, 347–351
- Shepherd, P. R., Withers, D. J. and Siddle, K. (1998) Specificity in ligand binding and intracellular signalling by insulin and insulin-like growth factor receptors. *Biochem. J.* **333**, 471–490
- Saltiel, A. R. and Kahn, C. R. (2001) Insulin signalling and the regulation of glucose and lipid metabolism. *Nature (London)* **414**, 799–806
- Siddle, K., Urso, B., Niesler, C. A., Cope, D. L., Molina, L., Surinya, K. H. and Soos, M. A. (2001) Specificity in ligand binding and intracellular signalling by insulin and insulin-like growth factor receptors. *Biochem. Soc. Trans.* **29**, 513–525
- White, M. F. (1997) The insulin signalling system and the IRS proteins. *Diabetologia* **40** (suppl. 2), S2–S17
- White, M. F. (2002) IRS proteins and the common path to diabetes. *Am. J. Physiol. Endocrinol. Metab.* **283**, 413–422
- Zick, Y. (2001) Insulin resistance: a phosphorylation-based uncoupling of insulin signaling. *Trends Cell Biol.* **11**, 437–441
- Johnston, A. M., Pirola, L. and Van Obberghen, E. (2003) Molecular mechanisms of insulin receptor substrate protein-mediated modulation of insulin signalling. *FEBS Lett.* **546**, 32–36
- Tang, X., Batty, I. H. and Downes, C. P. (2002) Muscarinic receptors mediate phospholipase C-dependent activation of protein kinase B via Ca^{2+} , ErbB3 and phosphoinositide 3-kinase in 1321N1 astrocytoma cells. *J. Biol. Chem.* **277**, 338–344
- Tang, X., Wang, L., Proud, C. G. and Downes, C. P. (2003) Muscarinic receptor-mediated activation of p70 S6 kinase 1 (S6K1) in 1321N1 astrocytoma cells: permissive role of phosphoinositide 3-kinase. *Biochem. J.* **374**, 137–143
- Alessi, D. R., Cohen, P., Ashworth, A., Cowley, S., Leeyers, S. J. and Marshall, C. J. (1995) Assay and expression of mitogen-activated protein kinase, MAP kinase kinase and Raf. *Methods Enzymol.* **255**, 279–290
- Batty, I. H. and Downes, C. P. (1994) The inhibition of phosphoinositide synthesis and muscarinic-receptor-mediated phospholipase C activity by Li^+ as secondary, selective, consequences of inositol depletion in 1321N1 cells. *Biochem. J.* **297**, 529–537
- Herbert, T. P., Kilhams, G. R., Batty, I. H. and Proud, C. G. (2000) Distinct signalling pathways mediate insulin and phorbol ester-stimulated eukaryotic initiation factor 4F assembly and protein synthesis in HEK 293 cells. *J. Biol. Chem.* **275**, 11249–11256
- Ashkenazi, A., Peralta, E. G., Winslow, J. W., Ramachandran, J. and Capon, D. J. (1989) Functional diversity of muscarinic receptor subtypes in cellular signal transduction and growth. *Trends Pharmacol. Sci. (Dec. suppl.)*, 16–22
- Nakahata, N. and Harden, T. K. (1987) Regulation of inositol trisphosphate accumulation by muscarinic cholinergic and H_1 -histamine receptors on human astrocytoma cells. Differential induction of desensitization by agonists. *Biochem. J.* **241**, 337–344
- Nakahata, N., Martin, M. W., Hughes, A. R., Hepler, J. R. and Harden, T. K. (1986) H_1 -histamine receptors on human astrocytoma cells. *Mol. Pharmacol.* **29**, 188–195
- Harden, T. K., Foster, S. J. and Perkins, J. P. (1979) Differential expression of components of the adenylate cyclase system during growth of astrocytoma cells in culture. *J. Biol. Chem.* **254**, 4416–4422
- Batty, I. H. and Downes, C. P. (1995) The mechanism of muscarinic receptor-stimulated phosphatidylinositol resynthesis in 1321N1 astrocytoma cells and its inhibition by Li^+ . *J. Neurochem.* **65**, 2279–2289
- Batty, I. H. and Nahorski, S. R. (1989) Rapid accumulation and sustained turnover of inositol phosphates in cerebral-cortex slices after muscarinic-receptor stimulation. *Biochem. J.* **260**, 237–241

- 28 Hughes, A. R. and Putney, Jr, J. W. (1989) Source of ^3H -labeled inositol bis- and monophosphates in agonist-activated rat parotid acinar cells. *J. Biol. Chem.* **264**, 9400–9407
- 29 Davies, S. P., Reddy, H., Caivano, M. and Cohen, P. (2000) Specificity and mechanism of action of some commonly used protein kinase inhibitors. *Biochem. J.* **351**, 95–105
- 30 Lazareno, S., Popham, A. and Birdsall, N. J. (1998) Muscarinic interactions of bisindolylmaleimide. *Eur. J. Pharmacol.* **360**, 281–284
- 31 Martinson, E. A., Trillivas, I. and Brown, J. H. (1990) Rapid protein kinase C-dependent activation of phospholipase D leads to delayed 1,2-diglyceride accumulation. *J. Biol. Chem.* **265**, 22282–22287
- 32 Purkiss, J. R. (1996) Protein kinase C modulation of phosphoinositidase C in 1321N1 human astrocytoma cells. *Biochem. Soc. Trans.* **24**, 54S
- 33 Downing, G. J., Kim, S., Nakanishi, S., Catt, K. J. and Balla, T. (1996) Characterization of a soluble adrenal phosphatidylinositol 4-kinase reveals wortmannin sensitivity of type III phosphatidylinositol kinases. *Biochemistry* **35**, 3587–3594
- 34 Wang, L. and Proud, C. G. (2002) Ras/Erk signaling is essential for activation of protein synthesis by G_q protein-coupled receptor agonists in adult cardiomyocytes. *Circ. Res.* **91**, 821–829
- 35 Fantus, I. G. and Tsiani, E. (1998) Multifunctional actions of vanadium compounds on insulin signaling pathways: evidence for preferential enhancement of metabolic versus mitogenic effects. *Mol. Cell. Biochem.* **182**, 109–119
- 36 Morinville, A., Maysinger, D. and Shaver, A. (1998) From Vanadis to Atropos: vanadium compounds as pharmacological tools in cell death signalling. *Trends Pharmacol. Sci.* **19**, 452–460
- 37 Aguirre, V., Uchida, T., Yenush, L., Davis, R. and White, M. F. (2000) The c-Jun NH_2 -terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of Ser 307 . *J. Biol. Chem.* **275**, 9047–9054
- 38 Aguirre, V., Werner, E. D., Giraud, J., Lee, Y. H., Shoelson, S. E. and White, M. F. (2002) Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. *J. Biol. Chem.* **277**, 1531–1537
- 39 Rui, L., Aguirre, V., Kim, J. K., Shulman, G. I., Lee, A., Corbould, A., Dunaif, A. and White, M. F. (2001) Insulin/IGF-1 and $\text{TNF-}\alpha$ stimulate phosphorylation of IRS-1 at inhibitory Ser307 via distinct pathways. *J. Clin. Invest.* **107**, 181–189
- 40 Ozes, O. N., Akca, H., Mayo, L. D., Gustin, J. A., Maehama, T., Dixon, J. E. and Donner, D. B. (2001) A phosphatidylinositol 3-kinase/Akt/mTOR pathway mediates and PTEN antagonizes tumor necrosis factor inhibition of insulin signaling through insulin receptor substrate-1. *Proc. Natl. Acad. Sci. U.S.A.* **98**, 4640–4645
- 41 Staubs, P. A., Nelson, J. G., Reichart, D. R. and Olefsky, J. M. (1998) Platelet-derived growth factor inhibits insulin stimulation of insulin receptor substrate-1-associated phosphatidylinositol 3-kinase in 3T3-L1 adipocytes without affecting glucose transport. *J. Biol. Chem.* **273**, 25139–25147
- 42 De Fea, K. and Roth, R. A. (1997) Modulation of insulin receptor substrate-1 tyrosine phosphorylation and function by mitogen-activated protein kinase. *J. Biol. Chem.* **272**, 31400–31406
- 43 Li, J., DeFea, K. and Roth, R. A. (1999) Modulation of insulin receptor substrate-1 tyrosine phosphorylation by an Akt/phosphatidylinositol 3-kinase pathway. *J. Biol. Chem.* **274**, 9351–9356
- 44 MacKintosh, C. and MacKintosh, R. W. (1994) Inhibitors of protein kinases and phosphatases. *Trends Biochem. Sci.* **19**, 444–448
- 45 Mothe, I. and Van Obberghen, E. (1996) Phosphorylation of insulin receptor substrate-1 on multiple serine residues, 612, 632, 662 and 731, modulates insulin action. *J. Biol. Chem.* **271**, 11222–11227
- 46 Inoue, G., Cheatham, B., Emkey, R. and Kahn, C. R. (1998) Dynamics of insulin signaling in 3T3-L1 adipocytes. Differential compartmentalization and trafficking of insulin receptor substrate (IRS)-1 and IRS-2. *J. Biol. Chem.* **273**, 11548–11555
- 47 Lin, H. Y., Ballou, L. M. and Lin, R. Z. (2003) Stimulation of the $\alpha 1\text{A}$ adrenergic receptor inhibits PDGF-induced PDGF β receptor Tyr751 phosphorylation and PI 3-kinase activation. *FEBS Lett.* **540**, 106–110
- 48 Yagle, K., Lu, H., Guizzetti, M., Moller, T. and Costa, L. G. (2001) Activation of mitogen-activated protein kinase by muscarinic receptors in astroglial cells: role in DNA synthesis and effect of ethanol. *Glia* **35**, 111–120
- 49 Hernandez, M., Bayon, Y., Sanchez Crespo, M. and Nieto, M. L. (1997) Thrombin produces phosphorylation of cytosolic phospholipase A_2 by a mitogen-activated protein kinase kinase-independent mechanism in the human astrocytoma cell line 1321N1. *Biochem. J.* **328**, 263–269
- 50 Lavie, Y. and Agranoff, B. W. (1996) Carbachol inhibits insulin-stimulated phosphatidylinositol 3-kinase activity in SH-SY5Y neuroblastoma cells. *J. Neurochem.* **67**, 1245–1251
- 51 Trillivas, I., McDonough, P. M. and Brown, J. H. (1991) Dissociation of protein kinase C redistribution from the phosphorylation of its substrates. *J. Biol. Chem.* **266**, 8431–8438
- 52 Obara, Y., Kobayashi, H., Ohta, T., Ohizumi, Y. and Nakahata, N. (2001) Scabronine G-methylester enhances secretion of neurotrophic factors mediated by an activation of protein kinase C- ζ . *Mol. Pharmacol.* **59**, 1287–1297
- 53 Liu, Y. F., Paz, K., Herschkovitz, A., Alt, A., Tennenbaum, T., Sampson, S. R., Ohba, M., Kuroki, T., LeRoith, D. and Zick, Y. (2001) Insulin stimulates PKC ζ -mediated phosphorylation of insulin receptor substrate-1 (IRS-1). A self-attenuated mechanism to negatively regulate the function of IRS proteins. *J. Biol. Chem.* **276**, 14459–14465
- 54 Ravichandran, L. V., Esposito, D. L., Chen, J. and Quon, M. J. (2001) Protein kinase C- ζ phosphorylates insulin receptor substrate-1 and impairs its ability to activate phosphatidylinositol 3-kinase in response to insulin. *J. Biol. Chem.* **276**, 3543–3549

Received 6 November 2003/22 January 2004; accepted 9 February 2004

Published as BJ Immediate Publication 9 February 2004, DOI 10.1042/BJ20031700