Muscarinic receptors mediate a phospholipase C-dependent activation of protein kinase B via Ca\(^{2+}\), ErbB3 and phosphoinositide 3-kinase in 1321N1 astrocytoma cells.

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Running title: Muscarinic receptor activation of protein kinase B.
Summary:

In 1321N1 astrocytoma cells, heterotrimeric G-protein coupled receptors that activate phosphoinositide-specific phospholipase Cβ (PLCβ) isoforms via Gq, induced a prolonged activation of protein kinase B (PKB) after a short delay. For example, the effect of carbachol acting on M₃ muscarinic receptors is blocked by wortmannin, suggesting it is mediated via a phosphoinositide 3-kinase (PI 3-kinase). In support of this, carbachol increased PI 3-kinase activity in PI 3-kinase (p85) immunoprecipitates. The pathway linking PLC-coupled receptors to PI 3-kinase was deduced to involve phosphoinositide hydrolysis and Ca²⁺ dependent ErbB3 transactivation, but not protein kinase C (PKC), based on the following evidence. (i) Inhibition of carbachol stimulated PLC by pretreatment with the phorbol ester, PMA, concomitantly reduced PKB activity while stimulation of other PLC-coupled receptors also activated PKB. (ii) Ca²⁺ ionophores and thapsigargin stimulated PKB activity in a wortmannin-sensitive manner whilst BAPTA blocked carbachol-stimulated PKB activity. (iii) PMA alone did not activate PKB whilst a PKC inhibitor did not prevent the activation of PKB by carbachol. (iv) Carbachol stimulated ErbB3 tyrosine phosphorylation and association with the p85 and both these and PKB activity were blocked by AG1478, an EGFR tyrosine kinase inhibitor. These experiments define a novel pathway linking Gq-coupled GPCRs to the activation of PI 3-kinase and PKB.
Introduction

Protein kinase B (PKB), also known as c-Akt or Rac-PK is a 60 KDa serine/threonine kinase which is the cellular homologue of the viral oncogene, v-Akt (1, 2, 3). PKB family members appear to be over-expressed in some human cancers including those of the breast, pancreas and ovaries (3, 4). Much of the interest in PKB stems from the discovery that this kinase is rapidly activated in response to a wide variety of receptor tyrosine kinases (RTKs) and that such activation is prevented by strategies that inhibit or prevent the activation of phosphoinositide 3-kinases (PI 3-kinases; 2, 3, 5,6). PKB is now established as one of several direct targets of the lipid products of PI 3-kinases, phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P3) and PtdIns(3,4)P2 (7, 9) The current model for activation of PKB via RTKs involves phosphorylation at Thr-308 in the activation loop of the catalytic domain and Ser-473 in the carboxyl-terminal tail (5). The phosphorylation of both sites is required for maximum activation and requires an interaction between the amino-terminal pleckstrin homology (PH) domain of PKB with PtdIns(3,4,5)P3 or PtdIns(3,4)P2 (5, 10, 11). Lipid binding results in translocation of PKB to membranes and induces a conformational change in PKB making it an efficient substrate for upstream kinase(s). Phosphorylation of Thr-308 is accomplished by phosphoinositide-dependent protein kinase 1 (PDK1) which possesses a C-terminal PH domain that also binds PI 3-kinase lipid products. Phosphorylation of Ser-473 may involve PDK1 and, or a yet to be identified protein kinase, currently termed PDK2 (1, 5, 6, 7).

Recent reports have begun to implicate some G-protein coupled receptors in pathways that lead to the activation of PKB in nonhaemopoietic cells. These include Gi-coupled receptors in human embryonic kidney 293 cells, PC12 cells and the colon carcinoma...
cell line SW-480 (12, 13). Such responses are generally blocked by PI 3-kinase inhibitors (14, 15, 16) and have been proposed to involve direct regulation of PI 3-kinase \( \gamma \) by G-protein \( \beta\gamma \)-subunits (12, 14), even though endogenous expression of this isoform is thought to be restricted to haemopoietic cells (6, 13). Other studies have implicated the involvement of type IA PI3K through a variety of putative mechanisms including: (i) association with Ras (12); (ii) association with PKC\( \epsilon \) (13); (iii) direct interaction with G\( \beta\gamma \) (17); (iv) transactivation of EGF receptors (18). There is also at least one report of wortmannin-insensitive activation of PKB via Gs-coupled \( \beta\)-adrenergic receptors (19).

There are currently few reports of Gq-coupled receptors stimulating PKB and/or PI 3-kinases(20, 21) and our previous work established that such receptors actually reduce PI 3-kinase activity stimulated by insulin in 1321N1 astrocytoma cells (22). We were, therefore, surprised to observe substantial activation of PKB when these cells were exposed to the muscarinic cholinergic agonist, carbachol, which acts primarily on Gq-coupled M\( _3 \) receptors in this system (23). We show here that PKB activation occurs downstream of PLC activity, does not require PKC but does involve calcium, ErbB3 transactivation and subsequently activation of a p85-associated PI 3-kinase.
Experimental Procedures

**Materials:** 1321N1 cells were obtained from the European Tissue Culture Collection, and materials for cell culture were from Gibco or Sigma. Calcium ionophores (A23187, ionomycin), calcium chelator (acetoxyethyl-ester (BAPTA-AM)), thapsigargin, bisindolylmaleimide IX (RO-318220), phorbol-12-myristate-13-acetate (PMA) and pertussis toxin were from Calbiochem (UK). Antibodies to the following were obtained from the sources indicated: phosphotyrosine (Upstate Biotechnology Incorporated, UBI); insulin receptor substrate-1 (IRS-1, UBI or Santa Cruz), protein kinase B (PKB) isoforms (α, β and γ) and phospho-Ser473 PKB (MRC Protein Phosphorylation Unit, University of Dundee, Dundee, UK); phospho-Thr308 PKB antibodies were purchased from Cell Signalling (UK). Antibodies specific for the p85α subunit of PI 3-kinase were the generous gift of Professor M. Waterfield, University College London. Other materials were from sources defined previously (22).

**Cell Culture:** 1321N1 cells were grown to confluence in 6- or 24- multiwell plates as described previously (22).

**Cellular measurement of PLC or PI 3-kinase activity:** These activities were measured in cells in which the phosphoinositide pools were pre-labelled to steady-state with [3H]inositol (5-10µCi/ml,) exactly as described previously (22, 24).

**Preparation of cell lysates:** Cells were washed three times with Hepes-buffered, modified Krebs-Henseleit buffer (118mM NaCl, 4.69mM KCl, 1.18mM MgSO4, 1.29mM CaCl2, 1.18mM KH2PO4, 11.67mM glucose, 25mM Hepes, pH 7.4 at 37°C) and pre-incubated for 30min at 37°C in the same buffer prior to exposure to stimuli or appropriate vehicle. Where indicated, antagonists or inhibitors were present for all or
part of the 30min pre-incubation period as specified in the figure legends. Incubations were fixed by rapid removal of the incubation buffer and addition of ice-cold lysis buffer (1% v/v Triton X100, 120mM NaCl, 50mM NaF, 1mM MgCl₂, 1mM EGTA, 1mM EDTA, 5mM β-glycerophosphate, 25mM Hepes, pH 7.6 at 4°C, freshly supplemented with 0.1mM PMSF, 0.1mM benzamidine, 10µM leupeptin, 1mM Na₃VO₄ and 1 mM dithiothreitol). Cell debris was collected, transferred onto ice and subsequently centrifuged for 5min at 20,000g and 4°C to remove insoluble material. The supernatants from cell lysates were then either assayed directly as described below or frozen in liquid nitrogen and stored at -80°C for later analysis.

In vitro kinase assays:

PI 3-kinase: Cell lysates (~1mg protein) were mixed for >2hrs at 4°C on a shaking platform with antibodies against IRS-1 or p85 each of which was pre-coupled to protein-G beads (10µl/sample). Immunoprecipitates were collected by brief centrifugation then washed and assayed for PI 3-kinase activity as described previously (25, 26).

PKB assay: The PKB isoforms (α,β and γ) were immunoprecipitated from cell lysates (~300mg protein) using the isoform specific antibodies and the immunoprecipitates were assayed as described previously (2, 27) except that assays were continued for 45 min at 30°C.

SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblotting: SDS-PAGE and western blotting were performed as described previously (26).
Results:

Muscarinic receptor stimulation activates PKB: In previous work using 1321N1 cells to study cross-talk between PI 3-kinase and PLC-dependent signalling pathways we showed that insulin-stimulated production of PtdIns(3,4,5)P$_3$ was inhibited by agonists acting on PLC-coupled receptors. This results both from prolonged inhibition of insulin-stimulated PI 3-kinase activity and acute activation of an unidentified PtdIns(3,4,5)P$_3$ 5-phosphatase (22, 28). It was thus unexpected that PLC-coupled receptors would prove capable of stimulating PKB activity themselves. This activation is illustrated in Fig 1A in which PKB activity was analysed in immunoprecipitates from cells treated for different times with carbachol, a cholinergic agonist. Carbachol induced a time-dependent stimulation of PKB activity which showed a marked delay in onset of approximately 2 minutes and which reached a maximum of 4-fold above basal after 15 minutes. This response was due to activation of muscarinic receptors (presumable M$_3$ receptors since these predominate in this cell line (23)) and since the response was blocked by atropine (not shown). The effects of carbachol were relatively potent with an EC$_{50}$ of approximately 10µM (Fig 1B), a concentration approximately 2-3 fold lower than that required for half maximal activation of PLC in these cells. The majority of the PKB activity resulted from the α and γ isoforms while PKB β was barely detectable. PKB α and γ were activated to similar extents by carbachol (Fig1C).

Muscarinic receptor activation of PKB requires PI 3-kinase: As shown in Fig 2A, pre-treatment of 1321N1 cells with 100nM wortmannin abolished the carbachol-stimulated PKB response. In control experiments, wortmannin also blocked PKB
activation by insulin, which is known to regulate PKB via class 1A PI 3-kinases. The latter binds to phosphotyrosine-containing sequences in insulin receptor substrate (IRS) proteins via SH2 domain in the PI 3-kinase regulatory subunit. Fig 2A also shows that insulin activates PKB to a level approximately 3-fold greater than carbachol. The data in Fig 2B establishes that PKB activity correlates with the degree of phosphorylation of Ser473 and Thr-308, compatible with the hypothesis that PI 3-kinase dependent lipid products are responsible for the effects of both insulin and carbachol on PKB.

To address this question further we measured the levels of \[^{3}H\]PtdIns(3,4,5)P\(_3\) in \[^{3}H\]inositol labelled cells. Figure 3 shows that carbachol induced a limited but significant increase in \[^{3}H\]PtdIns(3,4,5)P\(_3\) of approximately 50% above basal (Fig 3A) which our earlier, preliminary study (22) would have easily overlooked. At maximally effective concentrations, insulin stimulates a much larger (≥10 fold) increase in PtdIns(3,4,5)P\(_3\) levels (22) in these cells. However, as shown in Fig 3B the dose curve for PI 3-kinase activation by insulin lies approximately 10-fold to the right of the corresponding dose curve for PKB activation. This implies that only a very small increase in PtdIns(3,4,5)P\(_3\) is required to achieve substantial activation of PKB.

The results illustrated in Fig 3C implicate a Type IA PI 3-kinase, at least in part, in the pathway leading to activation of PKB in response to carbachol. Stimulation of 1321N1 cells with carbachol for 15 minutes resulted in an approximately 3-fold increase in PI 3-kinase activity that could be immunoprecipitated with an anti-p85 antibody.

**Muscarnic-receptor activation of PKB requires PLC:** The muscarnic receptor-stimulated PKB response was unaltered after pre-incubation of the cells with pertussis
toxin, whereas adenosine stimulated PKB was abolished by this treatment. These results established that the muscarinic receptor mediated response is not mediated by Gi/o, but most probably, via Gq (Fig4A).

In addition to muscarinic receptors, 1321N1 cells express several other endogenous receptors which couple, most probably via Gq, to the stimulation of PLC. These include histamine H₁ receptors (29, 30), protease activated receptors (PAR) sensitive to thrombin (31) and the synthetic peptide ligand, SFLLRN (22), and bradykinin (BK) receptors (see Fig 4). Figure 4 shows that maximally effective concentrations of histamine, SFLLRN or BK each elicited activation of PKB in 1321N1 cells. The smaller effects of these stimuli on PKB activity compared with that of carbachol are generally compatible with their relative impacts on PLC activity over the time-scale shown. Direct, quantitative comparison of these events is difficult, however, since muscarinic receptor stimulation induces a persistent activation of PLC in 1321N1 cells while PLC responses to the other stimuli vary both in initial magnitude and in the rate and extent of desensitization (22, 29, 31). Indeed the comparative efficacy of carbachol and SFLLRN in activating PKB is particularly noteworthy as the initial (≤30sec) PLC response to the latter is several fold greater than that elicited by the former ligand, but desensitizes extensively within 1-2min following agonist addition (22) during which phase neither carbachol nor SFLLRN activates PKB. This result implies that relatively persistent stimulation of PLC is required for this response. However, the ability of thrombin receptors in 1321N1 cells to couple to G-proteins in addition to Gq (32, 33) may also be relevant in this regard.

Figure 4B also illustrates that, like PKB responses to carbachol, those to histamine, SFLLRN and BK are blocked by wortmannin, consistent again with a downstream requirement for PI 3-kinase. By contrast, stimulation of the endogenous β-adrenergic
receptor, which couples to the activation of adenyl cyclase in 1321N1 cells (34), did not enhance PKB activity (not shown). These data thus show a strong qualitative correlation between PLC and PKB activities compatible with the hypothesis that the latter can be activated in a PLC-dependent manner.

Previous work has shown that acute pretreatment of 1321N1 cells with phorbol ester markedly reduces maximal muscarinic receptor-stimulated PLC activity by uncoupling either the receptor/G-protein or the G-protein/PLC interaction (8). As illustrated in Figure 5, we took advantage of this to confirm that muscarinic receptor activation of PKB is dependent upon PLC activity. These data show a dose-related inhibition of carbachol-stimulated PLC activity by phorbol ester, with maximal inhibition of approximately 70% at 1µM PMA. These effects were paralleled by an almost identical degree of inhibition of the PKB response to carbachol. This inhibition could not have been mediated via a direct action on PKB as PMA had little or no effect on insulin-stimulated (Figure 5) or basal PKB activity (see below). Thus, these results strongly support the conclusion that PLC activity is required for the ability of muscarinic agonists to stimulate PKB activity.

**Ca^{2+} signals, but not protein kinase C, are required for carbachol-stimulated PKB activity:** The signalling pathways downstream of PLCs involve inositol trisphosphate-mediated Ca^{2+} release and diacylglycerol-stimulated PKC activity. Figure 6A examines the role of PKC in carbachol-stimulated PKB activity. Treatment of 1321N1 cells with the phorbol ester, PMA, on its own, failed to stimulate PKB. Consistent with this observation pre-treatment with the bisindolylmaleimide PKC inhibitor, Ro-318220, did not block carbachol-stimulated PKB (Fig 6A). On the contrary, Ro-
Ro-318220 enhanced both basal and carbachol-stimulated PKB activity, suggesting that either PKC or another Ro-318220-sensitive target exerts a tonic inhibitory influence on PKB activity in these cells.

By contrast, reagents that elevate cytosolic [Ca\(^{2+}\)] did stimulate PKB activity in a similar manner to carbachol. Thus Fig 6B shows stimulation of PKB activity by the Ca\(^{2+}\) ionophore, ionomycin, of a similar magnitude and time course to that induced by carbachol. The delayed response of 1-2 minutes is especially reminiscent of the receptor-mediated activation of PKB. Similar responses were also observed using another Ca\(^{2+}\) ionophore (A23187) and following application of a Ca\(^{2+}\) ATPase inhibitor, thapsigargin (Fig 6C), that raises cytosol [Ca\(^{2+}\)] by blocking its uptake into the endoplasmic reticulum (35). The effects of all three reagents were blocked by wortmannin suggesting that Ca\(^{2+}\) acts upstream of PI 3-kinase in this system.

If a Ca\(^{2+}\) signal is necessary and sufficient for the stimulation of PKB by carbachol, it would be predicted that the agonist-induced response should be inhibited by reagents that reduce cytosolic [Ca\(^{2+}\)]. Experiments addressing this issue are shown in Figure 6A in which intracellular [Ca\(^{2+}\)] was buffered by treating cells with the cell-penetrant acetoxymethylester of the Ca\(^{2+}\) chelator, BAPTA (BAPTA-AM). The results presented in Figure 6A show that carbachol-induced PKB activity was diminished in cells loaded with BAPTA-AM. As the PLC response to carbachol was not similarly affected by the chelator (not shown), these data clearly imply that the muscarinic receptor-mediated stimulation of PKB is dependent upon an increase in intracellular Ca\(^{2+}\) distal to PLC activation. We would note, however, that while BAPTA/AM always attenuated carbachol-stimulated PKB activity, its inhibitory effect was considerably reduced when presented to cells in culture medium containing serum.
**ErbB3 transactivation is required for carbachol-stimulated PKB:** As the above data suggest that PLC-mediated Ca\(^{2+}\) signals are capable of activating PKB we examined the effects of reagents that influence signalling cascades downstream of Ca\(^{2+}\). It has been reported that Ca\(^{2+}\) is required for EGFR transactivation in response to activation of some GPCRs. In the rat pheochromocytoma cell line, PC12, which is widely used to study neuronal differentiation, EGFR transactivation by either bradykinin or membrane depolarization was shown to be dependent on the presence of extracellular calcium (36). Recently, angiotensin II induced ERK activation was shown to be mediated by EGFR transactivation in a Ca\(^{2+}\) signal-dependent manner in cardiac fibroblasts (37), vascular smooth muscle cells (VSMC) (38) and liver epithelial cells (39). The EGFR transactivation itself was identified as a critical element in GPCR-induced mitogen signalling (40) and in regulation of various ion channels (41). We examined the possibility that PKB activation might be downstream of EGFR transactivation in response to carbachol. Carbachol, however, failed to stimulate a significant increase in EGFR tyrosine phosphorylation. In contrast, EGF stimulated EGFR tyrosine phosphorylation was readily detectable and could be blocked by preincubating the cells with the EGFR tyrosine kinase inhibitor, AG1478 (Fig7A).

By contrast, we found that another member of the erbB family, ErbB3, is tyrosine phosphorylated in response to both carbachol and EGF (Fig7B). The phosphorylation and activation of ErbB3 is known to generate a docking site for the SH2 domain of PI 3-kinase (42). As shown in Fig7B, the association of PI 3-kinase with ErbB3 increased significantly following stimulation with either carbachol or EGF, and the tyrosine phosphorylation of ErbB3 increased in a time dependent manner, with a lag phase of ~2 min, and reached maximum phosphorylation at ~15min. AG1478 inhibited both ErbB3 tyrosine phosphorylation and PKB activation (Fig 8A and B). Taken together
these data suggest that ErbB3 transactivation is required for activation of PKB by carbachol.
Discussion:

We have investigated the signalling pathway linking endogenous muscarinic cholinergic receptors to the activation of PKB in 1321N1 astrocytoma cells. The evidence implicates PLC-mediated phosphoinositide hydrolysis, Ca^{2+}, ErbB3 and the lipid products of PI 3-kinase as minimal components of the pathway. This is the first report directly demonstrating a link between a PLC-dependent signalling cascade and the activation of PKB, though other very recent studies have implied a similar conclusion (21).

There have been several recent reports of GPCR-mediated activation of PKB. The majority of these responses involved pertussis toxin-sensitive G-proteins and were inhibited by wortmannin suggesting a requirement for PI 3-kinase activity (12, 14, 15, 16). Relatively direct activation of the G-protein $\beta\gamma$-subunit sensitive PI 3-kinase $\gamma$ and p110$\beta$ isoforms have been suggested to account for some of these observations (12, 17), but evidence also exists for indirect mechanisms involving Ras (15) and transactivation of EGF receptors (18). By contrast, there is only one report of wortmannin-insensitive activation of PKB which was observed in $\beta$-adrenergic receptor-stimulated rat epididymal fat cells (19).

The involvement of PI 3-kinase lipid products in carbachol-stimulated PKB activation is indicated by the marked sensitivity of the response to wortmannin, by the detection of PKB Ser473 and Thr308 phosphorylation, by the enhanced PI 3-kinase activity of p85 immunoprecipitates and by the small, but detectable increase in PtdIns(3,4,5)P$_3$ concentration. It remains possible, however, that the effect of PI 3-kinase inhibitors in this report may be mediated by reduction of the basal level of 3-phosphoinositides
rather than by blockade of carbachol-induced PI 3-kinase activation. This seems unlikely because of the role of ErbB3 transactivation in carbachol-stimulated PKB activation and the inhibitory effect of AG1478, an EGF receptor tyrosine kinase inhibitor. The latter had no effect on basal PKB activity or PtdIns(3,4,5)P3 levels (not shown).

The carbachol-stimulated response seems unlikely to be the result of a direct activation of PI 3-kinase by G-protein subunits. Firstly, evidence in the literature suggests that endogenous expression of the class IB PI 3-kinase, p110\(\gamma/p101\) complex, is largely restricted to cells of haemopoietic lineage (6, 13). More recently, however, evidence has emerged suggesting that G\(\beta\gamma\) subunits can synergistically activate a Type IA PI 3-kinase in THP-1 cells stimulated with insulin, together with a stimulus for a pertussis toxin-sensitive GPCR (43). By transient transfection of PI 3-kinase isoforms into NIH3T3 cells, Murga et al (17) concluded that PI3K\(\beta\) expression is necessary and sufficient to activate PI3K-dependent pathways by GPCRs in cells lacking PI3K\(\gamma\), and that this involves the direct interaction of p110\(\beta\) with G\(\beta\gamma\) subunits. By contrast, however, we have shown that PLC-coupled thrombin receptors (22, or muscarinic receptors; Batty and Downes, unpublished data) profoundly inhibit insulin-stimulated PtdIns(3,4,5)P3 accumulation (28) in 1321N1 cells, suggesting that such a synergistic response or direct activation of PI3K by G\(\beta\gamma\) is not the means by which PKB is activated in these cells. This conclusion is supported by the lack of effect of pertussis toxin and by the kinetics of carbachol-stimulated PKB activity which was delayed in relation to insulin stimulated or other direct G-protein subunit stimulated PI 3-kinase responses (2, 12).
Experiments involving inhibition of agonist-stimulated PLC by pretreatment with phorbol esters or manipulation of cytosolic [Ca\(^{2+}\)] using a variety of reagents and the demonstration that stimulation of PKB is a common consequence of activating several PLC-coupled receptors, all strongly implicate PLC-dependent Ca\(^{2+}\) signals in the pathway leading to activation of PI 3-kinase/PKB. This conclusion is in accord with previously reported results obtained using cardiac myocytes (44). β-adrenergic receptor stimulation of such cells opens L-type voltage-sensitive Ca\(^{2+}\) channels and the resulting Ca\(^{2+}\) signal has been reported to mediate wortmannin-sensitive activation of PKB. Ca\(^{2+}\) ionophores and thapsigargin have also been shown to stimulate PKB in HEK-293 cells (45) suggesting that the pathway described here may be quite widespread. It has been reported that Ca\(^{2+}/\text{calmodulin}\) can directly activate PI 3-kinase (46), but this seems unlikely to explain the persistent elevation of PI 3-kinase activity we observed in well washed anti-p85 immunoprecipitates.

It has been reported that EGFR transactivation is a common element in GPCR signalling (40). Carbachol, however, failed to stimulate a significant increase of EGFR tyrosine phosphorylation, although AG1478 blocked carbachol stimulated PKB. This may reflect limited expression of EGFR in 1321N1 cells, but our data suggest that Ca\(^{2+}\) mediated PI 3-kinase activation involves an activation of ErbB3 and the subsequent recruitment of PI 3-kinase to the ErbB3 receptor. Since ErbB3 itself lacks intrinsic protein tyrosine kinase activity, it has been suggested that its phosphorylation occurs via heterodimerization with ErbB2 (47-49). The phosphorylation of ErbB2 has been reported in rat-1 cells stimulated by endothelin (ET-1), lysophosphatidic acid (LPA) and thrombin (40).
So far the precise route from Ca\(^{2+}\) to ErbB3 activation is unclear. One possibility could be the induction of secretion of EGF or other ligands for ErbB3 receptors compatible with the observed delay in the activation of PKB by carbachol. However, we were not able to detect activation of PKB using conditioned medium from cells pre-treated with carbachol, although this approach does not preclude release and action of EGF (or a similar ligand) in a tight autocrine loop. Recently it has been shown that EGF receptor transactivation by GPCRs involves proHB-EGF and a metalloproteinase activity that is rapidly induced upon GPCR-ligand interaction. The cleavage of the proHB-EGF by the metalloproteinase appeared to be essential for the GPCR-induced EGFR transactivation and downstream signalling (50). It has also been reported that Src plays a role in GPCR transactivation of EGFR (51), but this seems unlikely to explain the activation of PKB we observed in PP1 (a potent Src inhibitor) pre-incubated cells (not shown).

In summary, our data describes a novel mechanism coupling PLC-dependent receptors to activation of PKB. The signalling pathway involves phosphoinositide hydrolysis and the resulting Ca\(^{2+}\) signal which activates PI 3-kinase via ErbB3 transactivation. Very small increases in PI 3-kinase lipid products are sufficient to regulate the phosphorylation and hence activation of PKB. Due to the inherent lag in this response, only receptors that generate persistent Ca\(^{2+}\) signals are likely to cause significant activation of PKB.
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Figure legends:

Figure 1: **Muscarinic receptor activation stimulates PKB** (A) Time course: 1321N1 cells were incubated with 1mM carbachol for the times indicated and PKBα activity was then immunoprecipitated and assayed. (B) Concentration dependence: 1321N1 cells were incubated ± the indicated concentration of carbachol as indicated for 20 min. before measurement of PKBα activity as at (A). (C) PKB isoforms: 1321N1 cells were incubated ± 1mM carbachol for 15 min and the PKB isoforms α, β and γ were immunoprecipitated separately and assayed. The results are expressed as fold-stimulation over control values and represent the mean ± sem of five (A), six (B) and three (C) separate experiments.

Figure 2: **Carbachol-stimulated phosphorylation and activation of PKB is blocked by wortmannin:** Cells were pre-treated without (-) or with (+) wortmannin (100nM) for 15 min then further incubated as control (con) or with carbachol (carb, 1mM) or insulin (ins, 1µM) for 15min. Lyates were prepared and assayed for PKB activity (A) or samples (50µg of protein) analyzed by SDS-PAGE and immunoblotted for phospho-Ser473 PKB or phospho- Thr308 or total PKB (B). Data in figure (A) are the mean ± sem from 3 experiments. (B) shows samples from cells incubated as: 1+2, control: 3+4, carbachol, 5+6, insulin; without (1,3,5) or with (2,4,6) wortmannin. Similar results were obtained in 3 further experiments.

Figure 3: **Carbachol-stimulated PKB activity correlates with increased cellular PtdIns(3,4,5)P3 and PI 3-kinase activity.** (A) Cellular \[^{3}\text{H}]\text{PtdIns}(3,4,5)P_3 concentrations: Cells pre-labelled with \[^{3}\text{H}]\text{inositol (10}\mu\text{Ci/ml)} were incubated for
30 min as control (con) or with carbachol (1 mM, carb) before extraction and analysis of the [³H]metabolites. Results show the mean ± sem of triplicate values from a single experiment representative of 3 similar experiments. Carbachol- and insulin-stimulated increases in [³H]PtdIns(3,4,5)P₃ in each experiment were significant by Student’s ‘t’ test (P < 0.05). (B) The concentration-dependence for insulin-stimulation of PI 3-kinase vs PKB: Lysates from cells exposed to insulin (0-10 µM) for 15 min were assayed for PI 3-kinase or PKB activity present respectively in anti-IRS-1 or anti-PKB immunoprecipitates. Data represent the mean values of duplicate incubations in a single experiment representative of 3 similar experiments. (C). PI 3-kinase activity in anti-p85 immunoprecipitates. Cells were incubated for 15 min as indicated. Lysates were prepared and PI 3-kinase activity associated with anti-p85 immunoprecipitates were assayed. Results show the mean and range of duplicate values obtained in a single experiment representative of two similar experiments.

Figure 4: Multiple PLC-coupled receptors activate PKB via Gq and not Gi/o:
(A) Cells pre-incubated for 1 hr (+) pertussis toxin (P.T, 100 ng/ml) or (-) pertussis toxin for 1 hr were then treated as control (con) or stimulated with carbachol (carb, 1 mM) or adenosine (aden, 1 mM) for a further 15 min. Cells were then lysed and PKB activity assayed. Results represent the mean±sem of 3 experiments. (B) Cells pre-incubated for 15 min (+) or (-) wortmannin (WM, 100 nM) were then treated as control (con) or stimulated with carbachol (carb, 1 mM), bradykinin (BK, 10 µM) histamine (HA, 1 mM), thrombin receptor-activating peptide (SFLLRN, 100 µM) for a further 15 min. Cells were then lysed and PKB activity assayed. Results represent the mean±sem of 3 experiments.

Figure 5: PMA pre-treatment induces parallel inhibition of carbachol-stimulated PLC and PKB activities: Cells, either pre-labelled with [³H]inositol (5 µCi/ml) for the
measurement of PLC activity or unlabelled for assay of PKB, were pre-incubated for 15 min with PMA (0-1000 nM) then further incubated for 15 min with LiCl (10 mM) and either carbachol (1 mM) or insulin (1 μM). PKB activity was then measured in cell lysates as previously described and PLC activity measured by analysis of [3H]inositol phosphate accumulation. Insulin did not stimulate PLC activity. Results are the mean ± sem of 4 experiments.

Figure 6: Increased intracellular Ca^{2+} but not PKC activity mimics carbachol stimulation of PKB: (A) PKC, BAPTA/AM and RO-318220; Cells were pre-loaded for 60 min with BAPTA-AM (40 μM), in the absence of serum, or similarly treated with vehicle control (1%v/v DMSO) then transferred into incubation buffer and pre-incubated for 30 min before exposure to carbachol (1 mM) for 15 min or PMA (1 μM) for 15 min. Cells were lysed and PKB activity measured. Where present, RO-318220 was added 15 min prior to stimulate. Results are the mean ± sem of 3-8 separate experiments. (B+C) Ca^{2+}; (B) Cells were incubated as indicated ± ionomycin (1 μM), lysates made and assayed for PKB activity. Results are the mean ± sem of 5 experiments. (C) Cells were pre-incubated for 15 min ± wortmannin (WM, 100 nM) then treated for a further 15 min as control (con), or with ionomycin (IONO, 1 μM), A23187 (1 μM) or thapsigargin (THP, 1 μM) before lysis and assay of PKB. Results are the mean ± sem of 3 experiments.

Figure 7: Carbachol stimulates ErbB3 tyrosine phosphorylation and increased association with PI3K: (A) EGF but not carbachol stimulates EGFR tyrosine phosphorylation: Cells (6 well plates) were pre-incubated with AG1478 (100 nM) or treated with vehicle control (0.1%v/v DMSO) then transferred into incubation buffer
and pre-incubated for 30 min before exposure to carbachol (1 mM) or EGF (50 ng/ml) for 15 min. Cells were then lysed and immunoprecipitated with anti-EGFR antibodies and blotted with either anti-phosphotyrosine (4G10) or anti-EGFR antibodies. (B) ErbB3 tyrosine phosphorylation and association with PI3K: Cell lysates were immunoprecipitated with anti-ErbB3 antibodies and probed with either 4G10 or anti-ErbB3 antibodies. (C) Time course: 1321N1 cells were incubated with 1 mM carbachol for the times indicated and ErbB3 was then immunoprecipitated and probed with either anti-ErbB3, or 4G10 or p85 antibodies. Similar results were obtained in 2 further experiments (A) and 3 further experiments (B and C).

Figure 8: **Carbachol stimulated ErbB3 tyrosine phosphorylation and PKB activation are blocked by AG1478:** Cells (6 well plates) were pre-incubated with AG1478 (100 nM) or treated with vehicle control (0.1% v/v DMSO) then transferred into incubation buffer and pre-incubated for 30 min before exposure to carbachol (1 mM) for 15 min. Cells were then lysed, and PKB activity measured in anti-PKBα immunoprecipitates (A). (A). Alternatively, lysates were immunoprecipitated with anti-ErbB3 antibodies and blotted with either anti-phosphotyrosine (4G10) or anti-ErbB3 antibodies. Results are the mean ± sem of 3 experiments (A) or representative of two separate experiments (B).
Abbreviations
RTK, Receptor-tyrosine kinase; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PKC, protein kinase C; PAGE, polyacrylamide gel electrophoresis; WM, wortmannin; RO-318220, bisindolylmaleimide IX.

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**Figure 1**

(A) Graph showing PKB activity (fold of stimulation) over Time of stimulation (min): 0, 5, 10, 15, 20, and 25.

(B) Graph showing PKB activity (fold of stimulation) with CARB (µM) concentrations: 0.001, 0.01, 0.1, 1, 10, 100, and 1000.

(C) Bar graph comparing PKB activity (cpm) for PKBα, PKBβ, and PKBγ under control (con) and carb (carb) conditions.
PKB activity (fold of control)

A

WM

-CON

+CARB

INS

B

IB

-Phospho-Ser^{473} PKB

-Phospho-Thr^{308} PKB

-PKB
Fig 7

A

1 2 3 4 5 6

IP: EGFR
IB: PY

IP: EGFR
IB: EGFR

B

1 2 3

IP: ErbB3
IB: p85

IP: ErbB3
IB: PY

C

IP: ErbB3

IB: PY

IB: p85

IB: ErbB3

Time of stimulation (Min) 0 2 5 15 20

170 kDa

85 kDa

170 kDa
Fig 8

Panel A: PKB activity (fold of control) with Ag1478 + +

Panel B: Immunoprecipitation (IP) with ErbB3 and Immunoblotting (IB) with pY and ErbB3.
Muscarinic receptors mediate a phospholipase C-dependent activation of protein kinase B via Ca2+, ErbB3 and phosphoinositide 3-kinase in 1321N1 astrocytoma cells

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