Phosphorylation of the $G_{q/11}$-Coupled $M_3$-Muscarinic Receptor is Involved in Receptor Activation of the ERK-1/2 Mitogen Activated Protein Kinase Pathway

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Running Title:

$M_3$-Muscarinic receptor phosphorylation in ERK-1/2 activation.
Summary.

We investigated the role played by agonist-mediated phosphorylation of the $G_{q/11}$-coupled M$_3$-muscarinic receptor in the mechanism of activation of the mitogen activated protein kinase pathway, ERK-1/2, in transfected Chinese hamster ovary cells. A mutant of the M$_3$-muscarinic receptor, where residues Lys$^{370}$-Ser$^{425}$ of the third intracellular loop had been deleted, showed a reduced ability to activate the ERK-1/2 pathway. This reduction was evident despite the fact that the receptor was able to couple efficiently to the phospholipase C second messenger pathway. Importantly, the ERK-1/2 responses to both the wild type M$_3$-muscarinic receptor and $\Delta$Lys$^{370}$-Ser$^{425}$ receptor mutant were dependent on the activity of protein kinase C. Our results, therefore, indicate the existence of two mechanistic components to the ERK-1/2 response which appear to act in concert. Firstly, the activation of protein kinase C through the diacylglycerol arm of the phospholipase C signalling pathway and a second component, absent in the $\Delta$Lys$^{370}$-Ser$^{425}$ receptor mutant, that is independent of the phospholipase C signalling pathway.

The reduced ability of the $\Delta$Lys$^{370}$-Ser$^{425}$ receptor mutant to activate the ERK-1/2 pathway correlated with an ~80% decrease in the ability of the receptor to undergo agonist-mediated phosphorylation. Furthermore, we have previously shown that M$_3$-muscarinic receptor phosphorylation can be inhibited by a dominant negative mutant of casein kinase 1$\alpha$ and by expression of a peptide corresponding to the third intracellular loop of the M$_3$-muscarinic receptor. Expression of these inhibitors of receptor phosphorylation reduced the wild type M$_3$-muscarinic receptor ERK-1/2 response. We conclude that phosphorylation of the M$_3$-muscarinic receptor on sites in the third intracellular loop by casein kinase 1$\alpha$ contributes to the mechanism of receptor activation of ERK-1/2 by working in concert with the diacylglycerol/PKC arm of the phospholipase C signalling pathway.
Introduction.

It is now clear that mitogenic signals mediated by the mitogen-activated protein (MAP) kinases, ERK-1 and ERK-2, can be initiated by both receptor tyrosine kinases (RTKs) and by the heptahelical G-protein coupled receptors (GPCRs). The activation of the ERK-1/2 pathway by GPCRs is mediated by any one of a number of mechanisms (1) probably reflecting the diversity of receptors within this large gene family. These mechanisms appear quite distinct, for example, ERK-1/2 activation has been shown to proceed via a tyrosine kinase-dependent mechanism for some receptors and a tyrosine kinase-independent manner for others (2,3). Despite this diversity, common features do exist, the most prominent of which is that GPCRs activate ERK-1/2 by acting initially through “classical” heterotrimeric G-protein signalling pathways (4). For example, stimulation of ERK-1/2 by G_i-coupled receptors, such as m2-muscarinic, and α_2A-adrenergic receptors, is pertussis toxin-sensitive indicating a role of G_i-proteins (5-7). It is proposed that liberation of βγ-subunits from G_i-proteins is responsible for the initiation of tyrosine phosphorylation (3,8), possibly by the activation of Src or Src-like tyrosine kinases (9,10,11), that ultimately results in Ras-dependent ERK-1/2 activation (3,6,7,12).

Similarly, G_{q/11}-coupled receptors that stimulate phospholipase C and the subsequent hydrolysis of phosphatidylinositol 4,5 bisphosphate to produce the second messengers inositol (1,4,5) trisphosphate (Ins(1,4,5)P₃) and diacylglycerol, activate the ERK-1/2 pathway via G_{q/11}-heterotrimeric G-proteins. In this case there is evidence for the involvement of both βγ-subunits (6,13,14) and Gα_q/11-subunits (3,10,12,14,15). Furthermore, the activation of ERK-1/2 by these receptors appears to be dependent on PKC since inhibition of PKC either abolishes (3,15-17) or significantly diminishes (18-20) the ERK-1/2 response to G_{q/11}-coupled receptors. This is particularly apparent for the M₃-muscarinic receptor where the ERK-1/2 response is blocked by >85% by either PKC inhibition or PKC-down regulation (21-24).

Studies have also indicated that the Ca²⁺ mobilisation arm of the phospholipase C signalling pathway is important in the activation of ERK-1/2 by G_{q/11}-coupled receptors. Bradykinin, LPA (25) and α₁B-adrenergic (10) receptor-stimulated ERK-1/2 responses were shown to be dependent on changes in intracellular Ca²⁺. Receptor-mediated Ca^{2+}
mobilisation is proposed to activate the Ca\(^{2+}\)/PKC-sensitive tyrosine protein kinase, Pyk2 (26), which is thought to act up-stream of Ras in the Erk-1/2 pathway (10,25). In the case of receptors such as the angiotensin AT\(_1\) (27), bradykinin (28), CCK\(_A\) (18), chemokine CXC\(_R\)-1/2 (19) and purinergic P\(_{2Y2}\) receptors (20,29) the activation of ERK-1/2 is proposed to be via transactivation of RTKs, a process that is dependent on Ca\(^{2+}\) mobilisation and subsequent activation of Pyk2 or related kinases.

These studies indicate that the mechanism for G\(_{q/11}\)-coupled receptor-mediated ERK-1/2 activation is dependent on the coupling of the receptor to G\(_{q/11}\)-heterotrimeric G-proteins and subsequent phospholipase C signalling through Ca\(^{2+}\) mobilisation and PKC activation. A further component in the activation of the ERK-1/2 pathway by GPCRs has recently been suggested from studies on the \(\beta_2\)-adrenergic receptor where receptor phosphorylation has been shown to play a central role. The \(\beta_2\)-adrenergic receptor is phosphorylated by both PKA and the G-protein coupled receptor kinases (GRKs) (30). PKA phosphorylation of the receptor on sites on the third intracellular loop has been proposed to act as a “molecular switch” coupling the receptor to G-proteins and subsequently the activation of the ERK-1/2 pathway via the generation of \(\beta\gamma\)-subunits (31). The \(\beta_2\)-adrenergic receptor can also be phosphorylated in an agonist-dependent manner by the GRKs, particularly GRK-2. This has classically been considered to result in the recruitment of \(\beta\)-arrestin and receptor desensitisation (30). However, recent studies have shown that \(\beta\)-arrestin can act as an adaptor protein recruiting activated e-Src to the plasma membrane in a process that is essential in the activation of the ERK-1/2 pathway by the \(\beta_2\)-adrenergic receptor (32).

In the present paper we investigate the role played by receptor phosphorylation in the activation of the ERK-1/2 pathway by the G\(_{q/11}\)-coupled M\(_3\)-muscarinic receptor. This receptor is rapidly phosphorylated on serine following agonist occupation (33). However, in contrast to the \(\beta_2\)-adrenergic receptor which is phosphorylated by the GRKs, M\(_3\)-muscarinic receptors are phosphorylated in an agonist-dependent manner on sites in the third intracellular loop by casein kinase 1\(\alpha\) (CK1\(\alpha\)) (34,35). Deletion of a region of the third intracellular loop of the human M\(_3\)-muscarinic receptor (Lys\(^{370}\)-Ser\(^{425}\)) reduced receptor phosphorylation by \(\sim80\%\) (35). Furthermore, expression of a dominant negative mutant of CK1\(\alpha\) or a peptide corresponding to the third intracellular loop of the receptor,
reduced receptor phosphorylation (35). Using these reagents in the present study, we investigate the role played by agonist-mediated receptor phosphorylation in the activation of the ERK-1/2 pathway.

**Experimental Procedures**

**Cell culture.**

CHO cell lines were grown in medium consisting of αMEM supplemented with 10% foetal calf serum, 100 IU/ml penicillin, 100µg/ml streptomycin and 2.5µg/ml fungizone. Cells were grown in a 5% CO\textsubscript{2}/95% air, humidified incubator at 37\textdegree C. The ΔLys\textsuperscript{370}-Ser\textsuperscript{425} receptor mutant clone #2 was maintained in blasticidin (5µg/ml).

**Generation of the dominant negative mutant of CK1α (F-CK1αK46R).**

The dominant negative mutant of CK1α (F-CK1αK46R) was generated by point mutagenesis of the lysine residue at position 46, which represents the invariant lysine at the ATP binding site of CK1α. The lysine residue was mutated to an arginine as described previously (35).

**Generation of the third intracellular loop peptide (3i-loop peptide).**

The sequence encoding amino acids S\textsuperscript{345}-L\textsuperscript{463} from the third intracellular loop of the M\textsubscript{3}-muscarinic receptor was cloned into BamHI and EcoRI sites in pcDNA-3 (Invitrogen) as described previously (35).

**Generation of the M\textsubscript{3}-Muscarinic Receptor Deletion Mutant ΔLys\textsuperscript{370}-Ser\textsuperscript{425}.**

Two stably transfected CHO cell lines expressing the M\textsubscript{3}-muscarinic receptor deletion mutant ΔLys\textsuperscript{370}-Ser\textsuperscript{425} were used in the present study. Clone #1 was generated by digestion of the M\textsubscript{3}-muscarinic receptor coding sequence contained in pcDNA-3 (Invitrogen) with HindIII and then religating the plasmid. This removed the coding sequence for amino acids Lys\textsuperscript{370}-Ser\textsuperscript{425} inclusive but maintained the reading frame of the remaining cDNA. This construct was transfected into CHO cells and clones selected using medium supplemented with G-418 (200µg/ml). The second clone used (clone#2) originated from...
another transfection where the cDNA encoding the $\Delta$Lys$^{370}$-Ser$^{425}$ receptor mutant was subcloned into pcDNA-6 (Invitrogen). Clones from this transfection were selected using medium supplemented with blasticidin (5µg/ml).

**Transient Transfections of CHO cells.**

Cells were plated onto 6 well dishes 24 hours before transfection. Cells (15-20% confluent) were transfected with either 3µg of F-CK1αK46R or 3i-loop peptide per well using 8µl of Fugene 6 transfection reagent (Boehringer). Cells were used 48 hours after transfection. Using a green fluorescent protein construct we estimated that the transfection efficiency was ~70%.

**Quantification of M$_3$-Muscarinic Receptor Expression.**

M$_3$-Muscarinic receptor expression on intact plated-down cells was determined using a saturating concentration of the hydrophilic muscarinic antagonist $[^3]$H-N-methyl scopolamine ($[^3]$H-NMS, ~0.5nM) as described previously (35). Non-specific binding was determined in the presence of 20µM atropine and was < 3% of the total binding.

**Mass Ins(1,4,5)P$_3$ Determination.**

Cells grown in 24 well dishes were washed with Krebs/HEPES buffer (HEPES 10mM, NaCl 118mM, KHPO$_4$ 1.17mM, KCl 4.3mM, MgSO$_4$ 7 1.17mM, CaCl$_2$ 1.3mM, NaHCO$_3$ 25.0mM, glucose 11.7mM, (pH 7.4)) and challenged with agonist for the appropriate times. Incubations were terminated by rapid aspiration, and addition of ice-cold 0.5 M trichloroacetic acid and transfer to an ice-bath. After 15 min the supernatant was removed and neutralised by addition of EDTA and freon/tri-n-octylamine as described previously (36). Extracts were brought to pH 7 by addition of NaHCO$_3$ and stored at 4°C until analysis. Ins(1,4,5)P$_3$ mass measurements were performed using a radio-receptor assay described previously (37).

**Erk-1/2 assay.**

CHO cells grown to confluence in 6 well plates were serum-starved for 1 hour in Krebs/HEPES buffer and then stimulated with the appropriate agents. Stimulation’s were
terminated by aspiration and cells incubated for 10 min in lysis buffer (Tris 20mM, NP-40 0.5%, NaCl 250mM, EDTA 3mM, EGTA 3mM, PMSF 1mM, Na3VO4 1mM, DTT 1mM, benzamidine 5µg/ml (pH 7.6)) at 4°C. Solubilised CHO cell lysates were pre-cleared by centrifuging at 14000rpm for 5 min. Endogenous MAP kinase was immunoprecipitated using 0.2µg of anti-Erk-1/2 antiserum (Santa Cruz). Protein A sepharose immobilised MAP kinase was washed twice in lysis buffer and twice in assay buffer (HEPES 20mM, β-glycerophosphate 20mM, MgCl2 10mM, DTT 1mM, Na3VO4 50µM (pH 7.2)). Washed pellets were resuspended in assay buffer containing 2µCi [32P]ATP, 20µM ATP, 200µM EGFr (peptide encompassing region 661-681 of EGF receptor) and reactions left to proceed for 20 min at 37°C. Reactions were terminated by the addition of 25% TCA and spotted on to P81 phosphocellulose paper squares (Whatman). Squares were washed four times with 0.05% orthophosphoric acid and once with acetone and radioactivity associated with the EGFr determined by liquid scintillation counting.

**Determination of intracellular Ca2+ concentrations ([Ca2+]i).**

Confluent monolayers of cells in 175 cm² flasks were harvested and re-suspended in 2.5 ml of Krebs/HEPES buffer. A 0.5 ml aliquot of this was removed for determination of cellular autofluorescence. Fura-2-acetoxymethyl ester (Fura-2-AM: 5 µM) was added to the remaining 2 ml which was then left for approximately 40 min at room temperature with gentle mixing. Supernatant containing extracellular Fura-2-AM was removed following gentle centrifugation of 0.5 ml aliquots. Cells were resuspended in a cuvette containing 3 ml of Krebs/HEPES buffer at 37°C. Using a Perkin-Elmer LS-5B spectrofluorimeter with a cuvette water jacket to maintain the temperature at 37°C, emission at 509 nm was recorded following excitation at both 340 and 380 nm. The excitation ratio was recorded every 1s and converted to [Ca2+]i, as previously reported (38) using 0.1% Triton X-100 in the presence of a saturating [Ca2+] to determine Rmax and the addition of EGTA to determine Rmin. Cells were challenged with 10-50 µl of agonist. Initial experiments were conducted in the presence of 1.3 mM extracellular [Ca2+] (as represented in Figure 4b). In experiments to determine the potency of intracellular Ca2+ mobilisation by the full agonist methacholine (represented in Figure 4c) the experiments were conducted in Ca2+ free medium where the Krebs/HEPES buffer had been supplement with EGTA to reduce extracellular [Ca2+] to
approximately 100 nM (determined using Fura-2). This was to ensure that the ability of the agonist to mobilise intracellular Ca\(^{2+}\) stores was being measured since any changes in intracellular Ca\(^{2+}\) concentrations under these conditions would have been the result of release of Ca\(^{2+}\) from intracellular stores with no contribution being made from an influx of extracellular Ca\(^{2+}\).

**Results**

**ERK-1/2 activation by a phosphorylation deficient mutant of the M\(_3\)-muscarinic receptor.**

Previous studies from our laboratory and others have shown that M\(_3\)-muscarinic receptors activate the ERK-1/2 pathway in a PTX-insensitive, PKC-dependent manner (21-24). The time course for ERK-1/2 activation peaks at 5 minutes then falls to a plateau which is maintained for at least 20 minutes (21). In order to test whether receptor phosphorylation plays a role in the regulation of the ERK-1/2 pathway a mutant M\(_3\)-muscarinic receptor was used where residues Lys\(^{370}\)-Ser\(^{425}\) of the third intracellular loop of the human M\(_3\)-muscarinic receptor had been deleted. This mutant receptor, termed \(\Delta\text{Lys}^{370}\)-Ser\(^{425}\), had previously been demonstrated to show an \(~80\%\) decrease in its ability to undergo agonist-mediated phosphorylation (35). Two stably transfected CHO cell lines were prepared expressing the \(\Delta\text{Lys}^{370}\)-Ser\(^{425}\) receptor at levels comparable to the wild type controls (B\(_{\text{max}}\) values in fmoles of receptor/mg protein: wild type=908± 124, \(\Delta\text{Lys}^{370}\)-Ser\(^{425}\) mutant clone #1= 782± 67, mutant clone #2= 1209 ± 10).

Concentration-response analysis of CHO cells expressing the wild type M\(_3\)-muscarinic receptor (CHO-m3 cells) showed a receptor-mediated ERK-1/2 activation with a half maximal response (EC\(_{50}\)) to the agonist carbachol of 45 ± 1.3 nM (n=3, ± S.E.) (Figure 1.) This is very similar to the EC\(_{50}\) value which we obtained previously using another distinct M\(_3\)-muscarinic receptor transfected CHO cell line (21). In contrast to the wild type receptor, the mutant receptor showed a rightward shift in the ERK-1/2 concentration-response curve to carbachol (Figure 1). The EC\(_{50}\) values for the two clonal cell lines expressing the \(\Delta\text{Lys}^{370}\)-Ser\(^{425}\) mutant were 660 ± 100 nM and 300 ± 100 nM (n=3, ± S.E.) for clones #1 and #2, respectively. These EC\(_{50}\) values were significantly different from
the wild type receptor values (p<0.05, Student’s t test). In addition to a reduction in the potency of carbachol there was also a reduction in the maximal ERK-1/2 response with clone #1 showing a 23 ± 7% reduction and clone #2 a 61 ± 5% reduction (n=3, ± S.E.) in the maximal carbachol response compared to wild type receptor controls (Figure 1). The time course for activation of ERK-1/2 was not, however, significantly different between the control and mutant receptors (data not shown).

In order to test for the possibility of clonal variation between the wild type CHO-m3 cells and mutant receptor cell lines, concentration-response curves for serum-induced ERK-1/2 activation were carried out. The concentration-response curves for serum-activated ERK-1/2 in the mutant receptor CHO cell lines were not significantly different from that of the CHO-m3 cells (Figure 2). This indicated that there was no clonal difference in the ERK-1/2 pathway stimulated by serum.

PKC-dependence of muscarinic ERK-1/2 responses.

We have previously shown that the wild type M₃-muscarinic receptor-mediated ERK-1/2 response is dependent on PKC since inhibition of PKC using Ro-318220 or down regulation of PKC reduced the muscarinic-ERK-1/2 response by >90% (21). The ΔLys³⁷⁰-Ser⁴²⁵ receptor mutant response also appeared to be sensitive to PKC inhibition in a manner similar to the wild type receptor. The phorbol 12,13 dibutyrate ERK-1/2 responses in the CHO-m3 cells and cells expressing ΔLys³⁷⁰-Ser⁴²⁵ receptor mutant (clone #2) were completely inhibited by the PKC inhibitor Ro-318220 (Figure 3). The ERK-1/2 responses to carbachol in the CHO-m3 cells and the cells expressing the ΔLys³⁷⁰-Ser⁴²⁵ receptor mutant were inhibited (~90%) by Ro-318220 (Figure 3).

Coupling of the ΔLys³⁷⁰-Ser⁴²⁵ receptor mutant to the phospholipase C signalling pathway.

We have previously reported that the ΔLys³⁷⁰-Ser⁴²⁵ receptor mutant showed agonist and antagonist binding characteristics that were not significantly different from the wild type receptors (35). We have also reported that the ΔLys³⁷⁰-Ser⁴²⁵ receptor mutant is coupled to the phospholipase C pathway in a manner analogous to the wild type receptor.
For example, the time course of Ins(1,4,5)P$_3$ generation of both the wild type M$_3$-muscarinic receptor and the ΔLys$^{370}$-Ser$^{425}$ receptor mutant peaks within 5-10 seconds of agonist stimulation and reaches a plateau phase after 60 seconds which is maintained for at least 5 minutes (35). Significantly, we have shown previously that the ΔLys$^{370}$-Ser$^{425}$ receptor mutant appears to give a more robust Ins(1,4,5)P$_3$ response than the wild type receptor suggesting that the receptor may be more efficiently coupled to phospholipase C (35). In the present study this characteristic is evident by an ~3.0 fold greater production of Ins(1,4,5)P$_3$ at maximal agonist concentration (Figure 4a).

We have reported previously that despite the fact that the ΔLys$^{370}$-Ser$^{425}$ receptor mutant was able to drive a larger Ins(1,4,5)P$_3$ response, the potency of the full agonist carbachol to mediated an Ins(1,4,5)P$_3$ response was not significantly different between the mutant and wild type receptors which had EC$_{50}$ values of 9.71 ±1.9µM and 7.14 ±3.2µM (n=3, ± S.E.), respectively (35).

The ability of the ΔLys$^{370}$-Ser$^{425}$ receptor mutant (clone #1) to mobilise intracellular Ca$^{2+}$ was also tested. The time course for receptor-mediated increases in intracellular Ca$^{2+}$ for both mutant and wild type receptors were similar (Figure 4b). Interestingly, in contrast to the Ins(1,4,5)P$_3$ response, there was no significant difference in the magnitude of the Ca$^{2+}$ mobilisation response between the wild type and ΔLys$^{370}$-Ser$^{425}$ receptor mutant. Similarly, the concentration-response curves for peak Ca$^{2+}$ mobilisation were not significantly different with EC$_{50}$ values of 166 ± 70nM and 258 ± 50nM (n=3, ± S.E.) for the wild type M$_3$-muscarinic receptor and ΔLys$^{370}$-Ser$^{425}$ receptor mutant, respectively (Figure 4c). (Note; in these Ca$^{2+}$ mobilisation experiments the full agonist methacholine was used. Both methacholine and carbachol are full agonists at the M$_3$-muscarinic receptor and produce almost identical responses.)

**Effect of the CK1α-dominant negative mutant (F-CK1α-K46R) and the third intracellular loop peptide (3i-loop peptide) on M$_3$-muscarinic receptor-mediated ERK-1/2 activation.**

Our previous studies had shown that CK1α was able to phosphorylate the M$_3$-muscarinic receptor in an agonist-dependent manner (34). Furthermore, we demonstrated that transient expression of a dominant negative mutant of CK1α (F-CK1α-K46R) was
able to reduce receptor phosphorylation by ~40% (35). In these earlier studies we also showed that expression of a peptide corresponding to the third intracellular loop of the M₃-muscarinic receptor (Ser³⁴⁵-Leu⁴⁶³), named the 3i-loop peptide, resulted in inhibition of receptor phosphorylation by >70% (35). To test the role that receptor phosphorylation might play in ERK-1/2 activation we transiently transfected F-CK1α-K46R and the 3i-loop peptide into CHO-m3 cells stably expressing the M₃-muscarinic receptor. Expression of F-CK1α-K46R and the 3i-loop peptide resulted in the reduction of the carbachol-mediated ERK-1/2 response by 53.9 ± 7.7% and 49.4 ± 3.2%, respectively (Figure 5a).

Control experiments were designed to test the ability of F-CK1α-K46R or the 3i-loop peptide to inhibit non-receptor mediated ERK-1/2 activation. Hence, the effect of transient transfection of F-CK1α-K46R or the 3i-loop peptide on the phorbol 12,13 dibutyrate ERK-1/2 response in native CHO-K1 cells was tested. It was found that neither F-CK1α-K46R or the 3i-loop peptide had any significant effect on the phorbol ester-mediated ERK-1/2 response in these cells (Figure 5b).

It is interesting to note that in experiments where phorbol esters were used to stimulate ERK-1/2 activity in CHO-m3 cells the F-CK1α-K46R construct inhibited the phorbol ester response by 31% (data not shown). The fact that the F-CK1α-K46R construct had very little effect on the phorbol ester response in CHO-K1 cells but a significant effect in CHO-m3 cells would suggest that in CHO-m3 cells the M₃-muscarinic receptor itself might contribute to the phorbol ester ERK-1/2 response. This may be due to the fact that phorbol esters are able to mediate phosphorylation of the agonist-unoccupied M₃-muscarinic receptor (33). This and other possibilities are presently under investigation.

Analysis of the ERK-1/2 concentration-response curves to carbachol demonstrated that in addition to reducing the maximal response the 3i-loop peptide and F-CK1α-K46R significantly (p<0.05, Student’s t test) reduced the potency of carbachol by 15.7 fold and 1.8 fold, respectively (Figure 6).
Discussion

Despite intensive research, the mechanisms employed by GPCRs in the activation of the ERK-1/2 pathway are generally poorly understood. One reason for this is that GPCRs are able to employ a number of diverse mechanisms in the activation of ERK-1/2 depending on the receptor type and the cellular environment (4). For example, m1- muscarinic receptor ERK-1/2 responses have been shown to operate in both a Ras-dependent (6) and Ras-independent (3) fashion using a mechanism which in some cell types employs tyrosine phosphorylation (2) and in others acts in a tyrosine kinase-independent manner (3). To add a further level of complexity, it has now become clear that a number of Gq/11-coupled receptors can simultaneously employ at least two independent mechanisms to activate the ERK-1/2 pathway (20,24,39). Despite this diversity there is one over-riding common feature in the mechanisms employed by Gq/11-coupled receptors, namely, the involvement of the Gq/11-heterotrimeric G-proteins and the subsequent activation of the phospholipase C signalling pathway. Both the Ins(1,4,5)P3/Ca2+ mobilisation and diacylglycerol/PKC arms of the phospholipase C signalling pathway have been implicated to play a role, and in many instances appear to provide the primary signal that links receptor activation to the initiation of the ERK-1/2 pathway.

We have shown previously that Gq/11-coupled M3-muscarinic receptors expressed in CHO cells stimulate the ERK-1/2 pathway in a PKC-dependent manner (21). This was confirmed in the present study and is consistent with previous reports from other laboratories (22-24) and would suggest that activation of PKC by the M3-muscarinic receptor is sufficient to stimulate ERK-1/2. This conclusion could be applied to a large number of Gq/11-coupled receptors that show PKC-dependent activation of ERK-1/2, such as; prostaglandin F2α (15), P2Y2-purinergic (20,29), CCK (18), m1-muscarinic, α1-adrenergic (3) and bradykinin (17) receptors. Furthermore, the ability of phorbol esters to increase ERK-1/2 activity (40) provides evidence that simply stimulating PKC is sufficient to drive the activation of ERK-1/2.

Thus, one model for ERK-1/2 activation by Gq/11-coupled receptors, including the M3-muscarinic receptor, would be that receptor-mediated PKC activation is sufficient to provide the signal that elicits the ERK-1/2 response.
Our data, however, using the ΔLys\textsuperscript{370}-Ser\textsuperscript{425} M\textsubscript{3}-muscarinic receptor mutant would suggest that this simple model is not correct. Deletion of Lys\textsuperscript{370}-Ser\textsuperscript{425} in the third intracellular loop of the human M\textsubscript{3}-muscarinic receptor resulted in a reduction in the ability of the receptor to stimulate ERK-1/2 activity. This reduction was evident despite the fact that the receptor was efficiently coupled to the phospholipase C signalling pathway. In fact this study, consistent with our previous report (35), demonstrates that the ΔLys\textsuperscript{370}-Ser\textsuperscript{425} receptor mutant is more efficiently coupled to the phospholipase C pathway than the wild type receptor. This suggests that simply activating the diacylglycerol/PKC arm of the phospholipase C signalling pathway was not in itself sufficient to drive a full G\textsubscript{q/11}-coupled receptor ERK-1/2 response. It is interesting to note that the ERK-1/2 response mediated by the ΔLys\textsuperscript{370}-Ser\textsuperscript{425} receptor mutant was still sensitive to PKC inhibition. Thus, the ΔLys\textsuperscript{370}-Ser\textsuperscript{425} receptor mutant ERK-1/2 response still has an absolute requirement for the activation of PKC but appears to be unable to employ an additional mechanism that is independent of G\textsubscript{q/11}-activated phospholipase C signalling. This additional mechanism (labelled mechanism 2 in Figure 7) appears to act in concert with PKC to elicit a full ERK-1/2 response.

These data, therefore, propose a model that identifies two mechanisms in the activation of ERK-1/2 (Figure 7). Mechanism 1 is PKC-dependent and is absolutely required for ERK-1/2 activation but when operating alone is only able to mediate a partial ERK-1/2 response. Mechanism 2 is PKC-independent and although is unable to elicit an ERK-1/2 response when operating alone it is able to act in concert with mechanism 1 to give a full ERK-1/2 response.

The most prominent PKC-independent mechanism assigned to G\textsubscript{q/11}-coupled receptor activation of ERK-1/2 is via the activity of the Ca\textsuperscript{2+}-sensitive tyrosine kinase Pyk2 or related kinases (25). Ins(1,4,5)P\textsubscript{3}-dependent increases in intracellular Ca\textsuperscript{2+} has been demonstrated to stimulate Pyk2 activity resulting in “transactivation” of RTKs and subsequent activation of the ERK-1/2 pathway (18-20,27-29). We can, however, eliminate the involvement of this process in the explanation of the results obtained with the ΔLys\textsuperscript{370}-Ser\textsuperscript{425} receptor mutant for two reasons. Firstly, the muscarinic receptor ERK-1/2 response in CHO cells is independent of changes in intracellular Ca\textsuperscript{2+} (22) suggesting that Pyk2 is not involved in the M\textsubscript{3}-muscarinic receptor response in these cells. Secondly, GPCR
transactivation of RTKs via Pyk2 is a process that involves Ins(1,4,5)P₃-mediated increases in intracellular Ca²⁺ (26). Since the ΔLys³⁷⁰-Ser⁴²⁵ receptor couples efficiently to the phospholipase C pathway, stimulating Ca²⁺ mobilisation in an identical manner to the wild type receptor, the involvement of a Ca²⁺-sensitive mechanism would not explain the lack of responsiveness of this receptor mutant.

Hence, the data presented here identifies a novel component of the M₃-muscarinic receptor ERK-1/2 response that is independent of activation of the Gq/11/phospholipase C pathway and dispels the notion that Gq/11-coupled receptors mediate ERK-1/2 activation by solely stimulating PKC or activating tyrosine phosphorylation via Ins(1,4,5)P₃-dependent increases in intracellular Ca²⁺.

We next tested the possibility that the novel component of the M₃-muscarinic receptor ERK-1/2 response involved agonist-mediated phosphorylation of the receptor. Our earlier studies had shown that the M₃-muscarinic receptor is rapidly phosphorylated on serine in an agonist-dependent manner (33). Extensive studies by our group have identified CK1α as a cellular kinase able to phosphorylate the M₃-muscarinic receptor (also the m1-muscarinic receptor and rhodopsin) in an agonist-dependent manner (34,35,41,42). These studies established for the first time a mechanism for agonist-dependent phosphorylation of GPCRs that was distinct from that of the GRKs. During these studies we suggested that sites within the third intracellular loop of the M₃-muscarinic receptor were important for the phosphorylation of the receptor. To test this we generated the ΔLys³⁷⁰-Ser⁴²⁵ receptor mutant which lacked 8 potential serine phospho-acceptor sites and the putative CK1α binding site (His³⁷⁴-Val³⁹¹) (35). Consistent with our hypothesis, the ΔLys³⁷⁰-Ser⁴²⁵ receptor mutant was reduced in its ability to undergo agonist-dependent phosphorylation by ~80% (35).

The reduced ability of the ΔLys³⁷⁰-Ser⁴²⁵ receptor mutant to undergo agonist-mediated phosphorylation correlates with the reduction in the receptor ERK-1/2 response and suggests that there is a link between receptor phosphorylation and activation of the ERK-1/2 pathway. It is of course possible that deletion of residues Lys³⁷⁰-Ser⁴²⁵ removes a domain involved in the ERK-1/2 response but which is not connected with receptor phosphorylation. This, in itself is an intriguing possibility and one that is being actively tested in our laboratory at the moment. However, our data to date is consistent with the hypothesis
that phosphorylation of the M₃-muscarinic receptor is involved in the PKC-dependent activation of the ERK-1/2 pathway.

We further investigated the role of receptor phosphorylation in the M₃-muscarinic receptor-mediated ERK-1/2 response by inhibiting phosphorylation of the wild type receptor. We have previously demonstrated that inhibition of CK1α-mediated M₃-muscarinic receptor phosphorylation could be achieved using either a dominant negative mutant of CK1α, F-CK1α-K46R, or expression of a region of the third intracellular loop of the M₃-muscarinic receptor (3i-loop peptide) that acted as a pseudo-substrate for CK1α (35). In the present study, expression of these constructs resulted in rightward shift in the concentration-response curve for carbachol-mediated ERK-1/2 activation and a reduction in the maximal ERK-1/2 response. The effect of these inhibitors of receptor phosphorylation appeared to be specific for the M₃-muscarinic-mediated ERK-1/2 response since expression of these constructs in CHO-K1 cells did not greatly affect the phorbol ester-mediated ERK-1/2 response. Furthermore, previously we have shown that F-CK1α-K46R did not prevent the receptor from coupling to the phospholipase C pathway but in fact increased the ability of the receptor to activate phospholipase C (35). Thus, the depressed ERK-1/2 response observed in the presence of inhibitors of receptor phosphorylation is receptor specific and produces a response in the wild type receptor that is very similar to that observed for the phosphorylation deficient ΔLys³⁷⁰-Ser⁴²⁵ receptor mutant. These data suggest, therefore, that agonist-mediated phosphorylation of the M₃-muscarinic receptor contributes to the mechanism of ERK-1/2 activation.

This conclusion is supported by recent reports linking phosphorylation of the β₂-adrenergic receptor to the regulation of ERK-1/2 activity. PKA-mediated phosphorylation of the β₂-adrenergic receptor has been demonstrated to act as a “molecular switch” resulting in the coupling of the receptor to the ERK-1/2 pathway via G₂-protein βγ-subunits (31). Furthermore, agonist-mediated GRK-2 phosphorylation has been shown to recruit a β-arrestin:c-Src complex to the β₂-adrenergic receptor (32). Preventing the ability of β-arrestin to interact with c-Src inhibits β₂-adrenergic receptor-mediated ERK-1/2 activation, suggesting that recruitment of c-Src to the phosphorylated β₂-adrenergic receptor via β-arrestin is essential in the mechanism of activation of ERK-1/2 (32). Hence, the data we present here indicates that the M₃-muscarinic receptor, in common with the β₂-adrenergic
receptor, employs agonist-mediated receptor phosphorylation in the mechanism of activation of the ERK-1/2 pathway.

In conclusion, we propose that agonist-mediated receptor phosphorylation via CK1α initiates a process that acts in concert with PKC to mediate a full M₃-muscarinic receptor ERK-1/2 response (Figure 7). The exact nature of the mechanism initiated by receptor phosphorylation is presently unclear but appears not to involve G₉/₁₁ heterotrimeric G-proteins nor the activation of the phospholipase C second messenger signalling cascade. We are presently pursuing the possibility that phosphorylation of sites in the third intracellular loop of the M₃-muscarinic receptor recruits an adaptor protein that is important in the activation of the ERK-1/2 pathway in a manner analogous to β-arrestin:Src and the β₂-adrenergic receptor.
References.


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**Abbreviations.**

[Ca<sup>2+</sup>]<sub>i</sub>, intracellular calcium concentration; CK1α, casein kinase 1α; ERK, Extracellular-
regulated protein kinases; GPCR, G-protein coupled receptor; GRK, G-protein coupled receptor kinase; Ins(1,4,5)P$_3$, inositol (1,4,5) trisphosphate; MAP mitogen-activated protein; PDBU, phorbol 12, 13 dibutyrate; PKC, protein kinase C. RTK, receptor-tyrosine kinases.

**Legends**

Fig. 1. *Activation of ERK-1/2 by wild type M$_3$-muscarinic receptors and the deletion mutant ΔLys$^{370}$-Ser$^{425}$. *Stably transfected CHO cells expressing either the wild type human M$_3$-muscarinic receptor or the deletion mutant ΔLys$^{370}$-Ser$^{425}$, were stimulated for 5 min. in the presence of varying concentrations of carbachol (CCH). The reaction was terminated by addition of lysis buffer and ERK-1/2 activity determined. Shown are the concentration-response curves for wild type receptor and two separate clones; (A) clone #1, (B) clone #2, expressing the ΔLys$^{370}$-Ser$^{425}$ receptor mutant. The data presented represents the mean ± S.E. for three experiments.

Fig. 2. *Serum-mediated ERK-1/2 responses.* Stably transfected CHO cells expressing either the wild type human M$_3$-muscarinic receptor or the deletion mutant ΔLys$^{370}$-Ser$^{425}$, were stimulated for 20 min. in the presence of varying concentrations of foetal calf serum. The reaction was terminated by addition of lysis buffer and ERK-1/2 activity determined. Shown are the concentration-response curves for wild type receptor and two separate clones; (A) clone #1, (B) clone #2, expressing the ΔLys$^{370}$-Ser$^{425}$ receptor mutant. The data represent the mean ± S.E. of at least three experiments.

Fig. 3. *PKC-dependency of ERK-1/2 responses.* Cells were pretreated with either
vehicle or the PKC-inhibitor Ro-318220 (10µM, Ro) for 10 mins. prior to stimulation with 1mM carbachol (CCH) or 1µM phorbol 12,13 dibutyrate (PDBu) or non-stimulated (Control). Stimulation’s were for 5 min. after which reactions were terminated by addition of lysis buffer and ERK-1/2 activity determined. The data represent the mean ± S.E. of three experiments.

Fig. 4. Coupling of the wild type M₃-muscarinic receptor and ΔLys⁴⁷⁰-Ser⁴²⁵ receptor mutant to the phospholipase C pathway. (A) Ins(1,4,5)P₃ generation was determined in cells that had been stimulated with carbachol (1mM) for varying times. The data represent the mean ± S.E. of three experiments carried out in duplicate. (B) Time-course of the changes in free intracellular Ca²⁺ concentrations ([Ca²⁺]ᵢ) determined in cell suspensions loaded with the Ca²⁺ indicator Fura2-AM and stimulated with the full agonist methacholine (1mM). (C) Concentration-response curve of the peak Ca²⁺ response following stimulation with methacholine. The data represent the mean ± S.E. of at least three experiments.

Fig. 5. Effect of the CK1α-dominant negative mutant (F-CK1αK46R) and the 3i-loop peptide on the M₃-muscarinic ERK-1/2 response. (A) CHO-m3 cells stably expressing recombinant M₃-muscarinic receptors or (B) native CHO-K1 cells, were transiently transfected with the CK1α-dominant negative mutant, F-CK1αK46R (K46R), or the 3i-loop peptide (3i-loop) corresponding to S₃⁴⁵-L₄⁶₃ of the third intracellular loop of the M₃-muscarinic receptor or were sham transfected (Control). 48 Hours after transfection, cells were stimulated with (A) 1mM carbachol (CCH) or (B) 1µM phorbol 12,13 dibutyrate (PDBu), for 5 min. Reactions were terminated using lysis buffer and ERK-1/2 activity determined. The data represent the mean ± S.E. of three experiments.

Fig. 6. ERK-1/2 concentration-response curves in CHO-m3 cells transiently transfected with the CK1α-dominant negative mutant (F-CK1αK46R) and the 3i-loop peptide. CHO-m3 cells stably expressing recombinant M₃-muscarinic receptors were transiently transfected with (A) the CK1α-dominant negative mutant, F-CK1αK46R (K46R), or (B) the 3i-loop peptide (3i-loop peptide) corresponding to S₃⁴⁵-L₄⁶₃ of the third intracellular loop of the M₃-muscarinic receptor. 48 Hours after transfection cells were
stimulated with varying concentrations of carbachol (CCH) for 5 min. Reactions were terminated using lysis buffer and ERK-1/2 activity determined. The data represent the mean ± S.E. of three experiments.

Fig. 7. Scheme of the mechanisms involved in the activation of the ERK-1/2 pathway by \(M_3\)-muscarinic receptors. Our data have identified two mechanisms involved in the activation of the ERK-1/2 pathway by \(M_3\)-muscarinic receptors expressed in CHO cells. Mechanism 1 is PKC-dependent and is essential in the activation of ERK-1/2. Inhibition of mechanism 1 (e.g., inhibition of PKC with Ro-318220) prevents activation of ERK-1/2 despite the fact that mechanism 2 is still intact. Mechanism 2, therefore, will not elicit an ERK-1/2 response alone. However, mechanism 2 does operate in concert with mechanism 1 to give a full ERK-1/2 response. Hence a receptor that is only able to activate mechanism 1 (i.e., the \(\Delta\text{Lys}^{70}\)-Ser\(^{425}\) receptor mutant or the wild type \(M_3\)-muscarinic receptor expressed together with the 3i-loop peptide or F-CK1\(\alpha\)K46R) will give a less than maximal ERK-1/2 response. Key: CK1\(\alpha\), casein kinase 1\(\alpha\); DAG, diacylglycerol; InsP\(_3\); inositol 1,4,5 trisphosphate; PIP\(_2\), phosphatidylinositol 4,5, bisphosphate; PLC, phospholipase C.
Figure 1
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A

- Log [CCH] vs. fmol phosphate incorporated / mg / min
- CHO-m3
- ΔLys<sup>370</sup>-Ser<sup>425</sup> Clone #1

B

- Log [CCH] vs. fmol phosphate incorporated / mg / min
- CHO-m3
- ΔLys<sup>370</sup>-Ser<sup>425</sup> Clone #2
Figure 2
Budd et al.

A

![Graph A]

B

![Graph B]

CHO-m3

\(\Delta\text{Lys}^{370}-\text{Ser}^{425}\) Clone #1

CHO-m3

\(\Delta\text{Lys}^{370}-\text{Ser}^{425}\) Clone #2

fmol phosphate incorporated / mg / min

% Serum

fmol phosphate incorporated / mg / min

% Serum
Figure 3
Budd et. al.

CHO-m3
ΔLys^{370}-Ser^{425}

fmol phosphate incorporated / mg / min
Figure 4
Budd et al.

A

\[
\text{pmol} \text{Ins}(1,4,5)P_3/\text{mg protein}
\]

\[
0 \hspace{1cm} 5 \hspace{1cm} 60
\]

CHO-m3
\[\Delta \text{Lys}^{370} \cdot \text{Ser}^{425}\]

B

\[
[\text{Ca}^{2+}]_i \ (\text{nM})
\]

\[
0 \hspace{1cm} 50 \hspace{1cm} 100 \hspace{1cm} 150 \hspace{1cm} 200
\]

CHO-m3
\[\Delta \text{Lys}^{370} \cdot \text{Ser}^{425}\]

C

\[
[\text{Ca}^{2+}]_i \ (\text{nM}) \text{ (Increase above basal)}
\]

\[
-9 \hspace{1cm} -8 \hspace{1cm} -7 \hspace{1cm} -6 \hspace{1cm} -5 \hspace{1cm} -4 \hspace{1cm} -3 \hspace{1cm} -2
\]

\[\log [\text{Methacholine}]\]

CHO-m3
\[\Delta \text{Lys}^{370} \cdot \text{Ser}^{425}\]
Figure 5

Budd et al.

A

CCH

- +

Control

K46R

3i-loop

fmol phosphate incorporated / mg / min

B

PDBu

- +

Control

K46R

3i-loop

fmol phosphate incorporated / mg / min
Figure 6
Budd et al.
Figure 7. Budd et al.
Phosphorylation of the Gq/11-coupled M3-muscarinic receptor is involved in receptor activation of the ERK-1/2 mitogen activated protein kinase pathway
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