

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*

Received for publication, September 27, 2000, and in revised form, November 9, 2000
Published, JBC Papers in Press, November 16, 2000, DOI 10.1074/jbc.M008827200

David C. Budd, Gary B. Willars, John E. McDonald, and Andrew B. Tobin‡

From the Department of Cell Physiology and Pharmacology, University of Leicester, P. O. Box 138, Medical Sciences Building, University Road, Leicester, LE1 9HN, United Kingdom

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 Lys³⁷⁰-Ser⁴²⁵ 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 Δ Lys³⁷⁰-Ser⁴²⁵ 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. First, the activation of protein kinase C through the diacylglycerol arm of the phospholipase C signaling pathway and a second component, absent in the Δ Lys³⁷⁰-Ser⁴²⁵ receptor mutant, that is independent of the phospholipase C signaling pathway. The reduced ability of the Δ Lys³⁷⁰-Ser⁴²⁵ 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 α 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 α contributes to the mechanism of receptor activation of ERK-1/2 by working in concert with the diacylglycerol/PKC arm of the phospholipase C signaling pathway.

It is now clear that mitogenic signals mediated by the mitogen-activated protein (MAP)¹ kinases, ERK-1 and ERK-2, can

* This work was supported by Wellcome Trust Grant No. 047600/Z/96. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed. Tel.: 0116-2522935; Fax: 0116-2523996; E-mail: TBA@le.ac.uk.

¹ The abbreviations used are: MAP, mitogen-activated protein; [Ca²⁺]_i, intracellular calcium concentration; CK1 α , casein kinase 1 α ; ERK, extracellular-regulated protein kinases; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor kinase; Ins(1,4,5)P₃, inositol (1,4,5)-trisphosphate; PKC, protein kinase C; PKA, cAMP-de-

pendent protein kinase; RTK, receptor-tyrosine kinases; GPCR, G-protein-coupled receptor. 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 signaling 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 $\beta\gamma$ -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 $\beta\gamma$ -subunits (6, 13, 14) and $G_{\alpha_{q/11}}$ -subunits (3, 10, 12, 14, 15). Furthermore, the activation of ERK-1/2 by these receptors appears to be dependent on PKC because 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 M_3 -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⁺ mobilization arm of the phospholipase C signaling pathway is important in the activation of ERK-1/2 by $G_{q/11}$ -coupled receptors. Bradykinin, LPA (25), and α_{1B} -adrenergic (10) receptor-stimulated ERK-1/2 responses were shown to be dependent on changes in intracellular Ca²⁺. Receptor-mediated Ca²⁺ mobilization is proposed to activate the Ca²⁺/PKC-sensitive tyrosine protein kinase, Pyk2 (26), which is thought to act upstream of Ras in the ERK-1/2 pathway (10, 25). In the case of receptors such as the angiotensin AT₁ (27), bradykinin (28), CCK_A (18), chemokine CXCR-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 Ca²⁺ mobilization and

pendent protein kinase; RTK, receptor-tyrosine kinases; CHO, Chinese hamster ovary cells.

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 signaling through Ca²⁺ mobilization 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 β₂-adrenergic receptor where receptor phosphorylation has been shown to play a central role. The β₂-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_i-proteins and subsequently the activation of the ERK-1/2 pathway via the generation of βγ-subunits (31). The β₂-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 β-arrestin and receptor desensitization (30). However, recent studies have shown that β-arrestin can act as an adaptor protein recruiting activated c-Src to the plasma membrane in a process that is essential in the activation of the ERK-1/2 pathway by the β₂-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 M₃-muscarinic receptor. This receptor is rapidly phosphorylated on serine following agonist occupation (33). However, in contrast to the β₂-adrenergic receptor, which is phosphorylated by the GRKs, M₃-muscarinic receptors are phosphorylated in an agonist-dependent manner on sites in the third intracellular loop by casein kinase 1α (CK1α) (34, 35). Deletion of a region of the third intracellular loop of the human M₃-muscarinic receptor (Lys³⁷⁰-Ser⁴²⁵) reduced receptor phosphorylation by ~80% (35). Furthermore, expression of a dominant negative mutant of CK1α 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% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml fungizone. Cells were grown in a 5% CO₂, 95% air, humidified incubator at 37 °C. The ΔLys³⁷⁰-Ser⁴²⁵ receptor mutant clone 2 was maintained in blasticidine (5 μg/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 Ser³⁴⁵-Leu⁴⁶³ from the third intracellular loop of the M₃-muscarinic receptor was cloned into BamHI and EcoRI sites in pcDNA-3 (Invitrogen) as described previously (35).

Generation of the M₃-Muscarinic Receptor Deletion Mutant ΔLys³⁷⁰-Ser⁴²⁵—Two stably transfected CHO cell lines expressing the M₃-muscarinic receptor deletion mutant ΔLys³⁷⁰-Ser⁴²⁵ were used in the present study. Clone 1 was generated by digestion of the M₃-muscarinic receptor coding sequence contained in pcDNA-3 (Invitrogen) with HindIII and then religating the plasmid. This removed the coding sequence for amino acids Lys³⁷⁰-Ser⁴²⁵ inclusive, but maintained the reading frame of the remaining cDNA. This construct was transfected into CHO cells, and clones were selected using medium supplemented with G-418 (200 μg/ml). The second clone used (clone 2) originated from another transfection where the cDNA encoding the ΔLys³⁷⁰-Ser⁴²⁵ receptor mutant was subcloned into pcDNA-6 (Invitrogen). Clones from this transfection were selected using medium supplemented with blasticidine (5 μg/ml).

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

Quantification of M₃-Muscarinic Receptor Expression—M₃-Muscarinic receptor expression on intact plated-down cells was determined using a saturating concentration of the hydrophilic muscarinic antagonist [³H]N-methyl scopolamine ([³H]NMS, ~0.5 nM) as described previously (35). Nonspecific binding was determined in the presence of 20 μM atropine and was < 3% of the total binding.

Mass Ins(1,4,5)P₃ Determination—Cells grown in 24-well dishes were washed with Krebs/HEPES buffer (HEPES (10 mM), NaCl (118 mM), KHPO₃ (1.17 mM), KCl (4.3 mM), MgSO₄·7 (1.17 mM), CaCl₂ (1.3 mM), NaHCO₃ (25.0 mM), glucose (11.7 mM), pH 7.4) and challenged with agonist for the appropriate times. Incubations were terminated by rapid aspiration, addition of ice-cold 0.5 M trichloroacetic acid, and transfer to an ice-bath. After 15 min, the supernatant was removed and neutralized by addition of EDTA and freon/tri-*n*-octylamine as described previously (36). Extracts were brought to pH 7 by addition of NaHCO₃ and stored at 4 °C until analysis. Ins(1,4,5)P₃ 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 h in Krebs/HEPES buffer and then stimulated with the appropriate agents. Stimulation was terminated by aspiration, and cells were incubated for 10 min in lysis buffer (Tris (20 mM), Nonidet P-40 (0.5%), NaCl (250 mM), EDTA (3 mM), EGTA (3 mM), phenylmethylsulfonyl fluoride (1 mM), Na₃VO₄ (1 mM), dithiothreitol (1 mM), benzamide (5 μg/ml), pH 7.6) at 4 °C. Solubilized CHO cell lysates were pre-cleared by centrifuging at 14,000 rpm for 5 min. Endogenous MAP kinase was immunoprecipitated using 0.2 μg of anti-Erk-1/2 antiserum (Santa Cruz). Protein A-Sepharose immobilized MAP kinase was washed twice in lysis buffer and twice in assay buffer (HEPES (20 mM), β-glycerophosphate (20 mM), MgCl₂ (10 mM), dithiothreitol (1 mM), Na₃VO₄ (50 μM), pH 7.2). Washed pellets were resuspended in assay buffer containing 2 μCi of [³²P]ATP, 200 μM ATP, 200 μM EGFr (peptide encompassing region 661–681 of the EGF receptor), and reactions were left to proceed for 20 min at 37 °C. Reactions were terminated by the addition of 25% trichloroacetic acid and spotted onto 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 was determined by liquid scintillation counting.

Determination of Intracellular Ca²⁺ Concentrations ([Ca²⁺]_i)—Confluent monolayers of cells in 175 cm² flasks were harvested and resuspended 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 ~40 min at room temperature with gentle mixing. Supernatant containing extracellular Fura-2-AM was removed following gentle centrifugation of the 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 1 s and converted to [Ca²⁺]_i as previously reported (38) using 0.1% Triton X-100 in the presence of a saturating [Ca²⁺]_o to determine R_{max} and the addition of EGTA to determine R_{min}. Cells were challenged with 10–50 μl of agonist. Initial experiments were conducted in the presence of 1.3 mM extracellular [Ca²⁺]_o (as represented in Fig. 4B). In experiments to determine the potency of intracellular Ca²⁺ mobilization by the full agonist methacholine (represented in Fig. 4C), the experiments were conducted in Ca²⁺-free medium where the Krebs/HEPES buffer had been supplemented with EGTA to reduce extracellular [Ca²⁺]_o to ~100 nM (determined using Fura-2). This was to ensure that the ability of the agonist to mobilize intracellular Ca²⁺ stores was being measured, because any changes in intracellular Ca²⁺ concentrations under these conditions would have been the result of release of Ca²⁺ from intracellular stores with no contribution being made from an influx of extracellular Ca²⁺.

RESULTS

ERK-1/2 Activation by a Phosphorylation-deficient Mutant of the M₃-Muscarinic Receptor—Previous studies from our lab

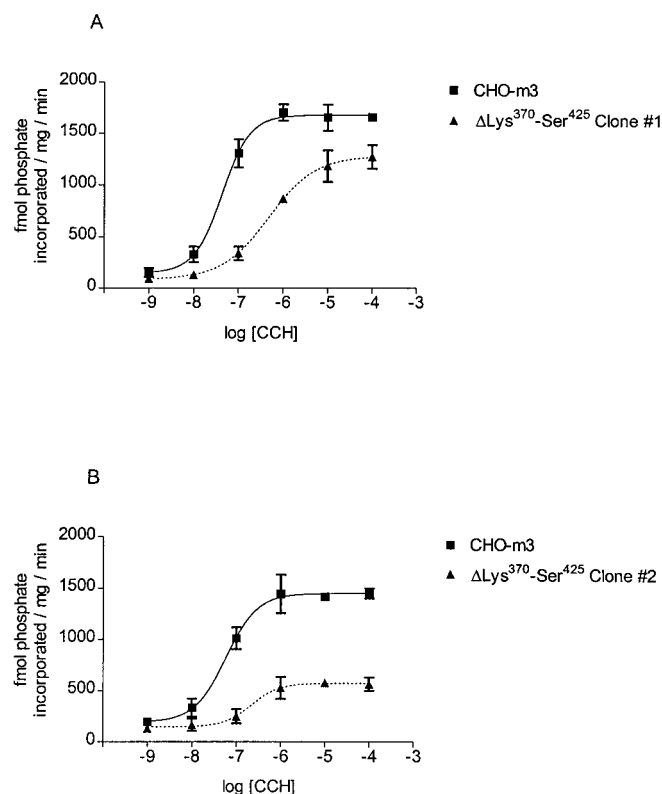


FIG. 1. Activation of ERK-1/2 by wild-type M₃-muscarinic receptors and the deletion mutant Δ Lys³⁷⁰-Ser⁴²⁵. Stably transfected CHO cells expressing either the wild-type human M₃-muscarinic receptor or the deletion mutant Δ Lys³⁷⁰-Ser⁴²⁵ were stimulated for 5 min in the presence of varying concentrations of carbachol (CCH). The reaction was terminated by addition of lysis buffer, and ERK-1/2 activity was determined. Shown are the concentration-response curves for wild-type receptor and two separate clones: clone 1 (A), clone 2 (B), expressing the Δ Lys³⁷⁰-Ser⁴²⁵ receptor mutant. The data presented represent the mean \pm S.E. for three experiments.

oratory and others (21–24) have shown that M₃-muscarinic receptors activate the ERK-1/2 pathway in a PTX-insensitive, PKC-dependent manner. The time course for ERK-1/2 activation peaks at 5 min then falls to a plateau, which is maintained for at least 20 min (21). To test whether receptor phosphorylation plays a role in the regulation of the ERK-1/2 pathway, a mutant M₃-muscarinic receptor was used where residues Lys³⁷⁰-Ser⁴²⁵ of the third intracellular loop of the human M₃-muscarinic receptor had been deleted. This mutant receptor, termed Δ Lys³⁷⁰-Ser⁴²⁵, had previously been demonstrated to show an \sim 80% decrease in its ability to undergo agonist-mediated phosphorylation (35). Two stably transfected CHO cell lines were prepared expressing the Δ Lys³⁷⁰-Ser⁴²⁵ receptor at levels comparable with the wild-type controls (B_{\max} values in fmols of receptor/mg protein: wild type = 908 \pm 124, Δ Lys³⁷⁰-Ser⁴²⁵ mutant clone 1 = 782 \pm 67, mutant clone 2 = 1209 \pm 10).

Concentration-response analysis of CHO cells expressing the wild-type M₃-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 \pm 1.3 nM ($n = 3$, \pm S.E., Fig. 1.) This is very similar to the EC_{50} value that we obtained previously using another distinct M₃-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 (Fig. 1). The EC_{50} values for the two clonal cell lines expressing the Δ Lys³⁷⁰-Ser⁴²⁵ mutant were 660 \pm 100 nM and 300 \pm 100 nM ($n = 3$, \pm S.E.) for clones 1 and 2, respectively. These EC_{50}

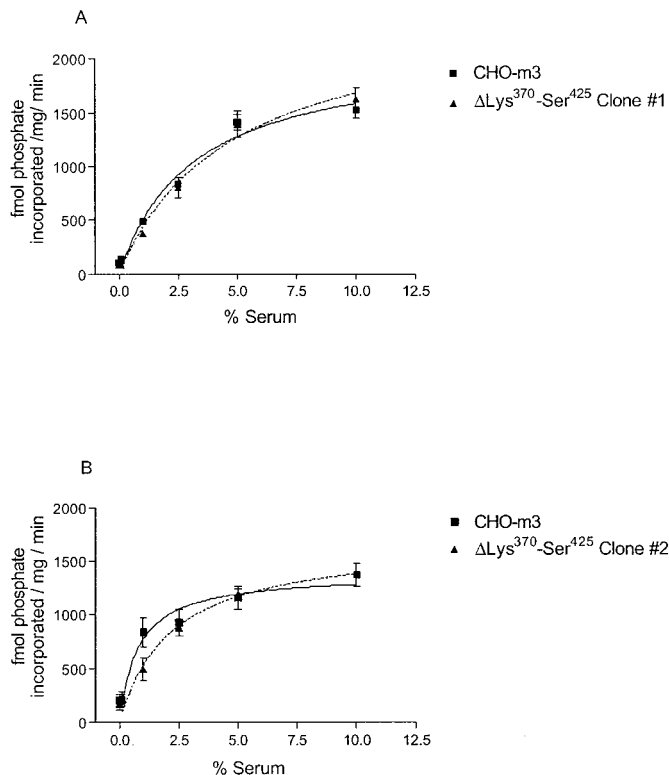


FIG. 2. Serum-mediated ERK-1/2 responses. Stably transfected CHO cells expressing either the wild-type human M₃-muscarinic receptor or the deletion mutant Δ Lys³⁷⁰-Ser⁴²⁵ were stimulated for 20 min in the presence of varying concentrations of fetal calf serum. The reaction was terminated by addition of lysis buffer, and ERK-1/2 activity was determined. Shown are the concentration-response curves for wild-type receptor and two separate clones: clone 1 (A), clone 2 (B), expressing the Δ Lys³⁷⁰-Ser⁴²⁵ receptor mutant. The data represent the mean \pm S.E. of at least three experiments.

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 \pm 7% reduction and clone 2 a 61 \pm 5% reduction ($n = 3$, \pm S.E.) in the maximal carbachol response compared with wild-type receptor controls (Fig. 1). The time course for activation of ERK-1/2 was not, however, significantly different between the control and mutant receptors (data not shown).

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 (Fig. 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 because inhibition of PKC using Ro-318220 or down-regulation of PKC reduced the muscarinic-ERK-1/2 response by $>90\%$ (21). The Δ Lys³⁷⁰-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 Δ Lys³⁷⁰-Ser⁴²⁵ receptor mutant (clone 2) were completely inhibited by the PKC inhibitor Ro-318220 (Fig. 3). The ERK-1/2 responses to carbachol in the CHO-m3 cells and the cells expressing the Δ Lys³⁷⁰-Ser⁴²⁵ receptor mutant were inhibited ($\sim 90\%$) by Ro-318220 (Fig. 3).

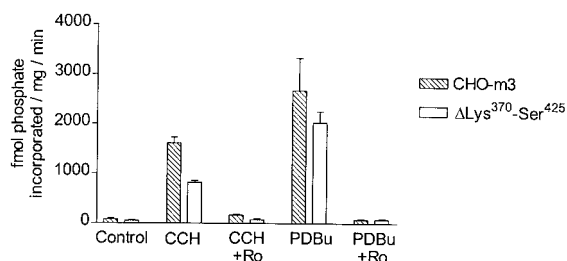


FIG. 3. PKC-dependence of ERK-1/2 responses. Cells were pretreated with either vehicle or the PKC-inhibitor Ro-318220 (10 μ M, Ro) for 10 min prior to stimulation with 1 mM carbachol (CCH) or 1 μ M phorbol 12,13-dibutyrate (PDBu) or nonstimulated (Control). Stimulation was for 5 min after which reactions were terminated by addition of lysis buffer, and ERK-1/2 activity was determined. The data represent the mean \pm S.E. of three experiments.

Coupling of the Δ Lys³⁷⁰-Ser⁴²⁵ Receptor Mutant to the Phospholipase C Signaling Pathway—We have previously reported that the Δ Lys³⁷⁰-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 Δ Lys³⁷⁰-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)P₃ generation of both the wild-type M₃-muscarinic receptor and the Δ Lys³⁷⁰-Ser⁴²⁵ receptor mutant peaks within 5–10 s of agonist stimulation and reaches a plateau phase after 60 s, which is maintained for at least 5 min (35). Significantly, we have shown previously that the Δ Lys³⁷⁰-Ser⁴²⁵ receptor mutant appears to give a more robust Ins(1,4,5)P₃ 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 \sim 3.0-fold greater production of Ins(1,4,5)P₃ at maximal agonist concentration (Fig. 4A).

We have reported previously that despite the fact that the Δ Lys³⁷⁰-Ser⁴²⁵ receptor mutant was able to drive a larger Ins(1,4,5)P₃ response, the potency of the full agonist carbachol to mediate an Ins(1,4,5)P₃ response was not significantly different between the mutant and wild-type receptors, which had EC₅₀ values of $9.71 \pm 1.9 \mu$ M and $7.14 \pm 3.2 \mu$ M ($n = 3$, \pm S.E.), respectively (35).

The ability of the Δ Lys³⁷⁰-Ser⁴²⁵ receptor mutant (clone 1) to mobilize intracellular Ca²⁺ was also tested. The time course for receptor-mediated increases in intracellular Ca²⁺ for both mutant and wild-type receptors were similar (Fig. 4B). Interestingly, in contrast to the Ins(1,4,5)P₃ response, there was no significant difference in the magnitude of the Ca²⁺ mobilization response between the wild-type and Δ Lys³⁷⁰-Ser⁴²⁵ receptor mutant. Similarly, the concentration-response curves for peak Ca²⁺ mobilization were not significantly different with EC₅₀ values of 166 ± 70 nM and 258 ± 50 nM ($n = 3$, \pm S.E.) for the wild-type M₃-muscarinic receptor and Δ Lys³⁷⁰-Ser⁴²⁵ receptor mutant, respectively (Fig. 4C). (Note; in these Ca²⁺ mobilization experiments the full agonist methacholine was used. Both methacholine and carbachol are full agonists at the M₃-muscarinic receptor and produce almost identical responses.)

Effect of the CK1 α Dominant Negative Mutant (F-CK1 α -K46R) and the 3i-Loop Peptide on M₃-Muscarinic Receptor-mediated ERK-1/2 Activation—Our previous studies had shown that CK1 α was able to phosphorylate the M₃-muscarinic receptor in an agonist-dependent manner (34). Furthermore, we demonstrated that transient expression of a dominant negative mutant of CK1 α (F-CK1 α -K46R) was able to reduce receptor phosphorylation by \sim 40% (35). In these earlier studies,

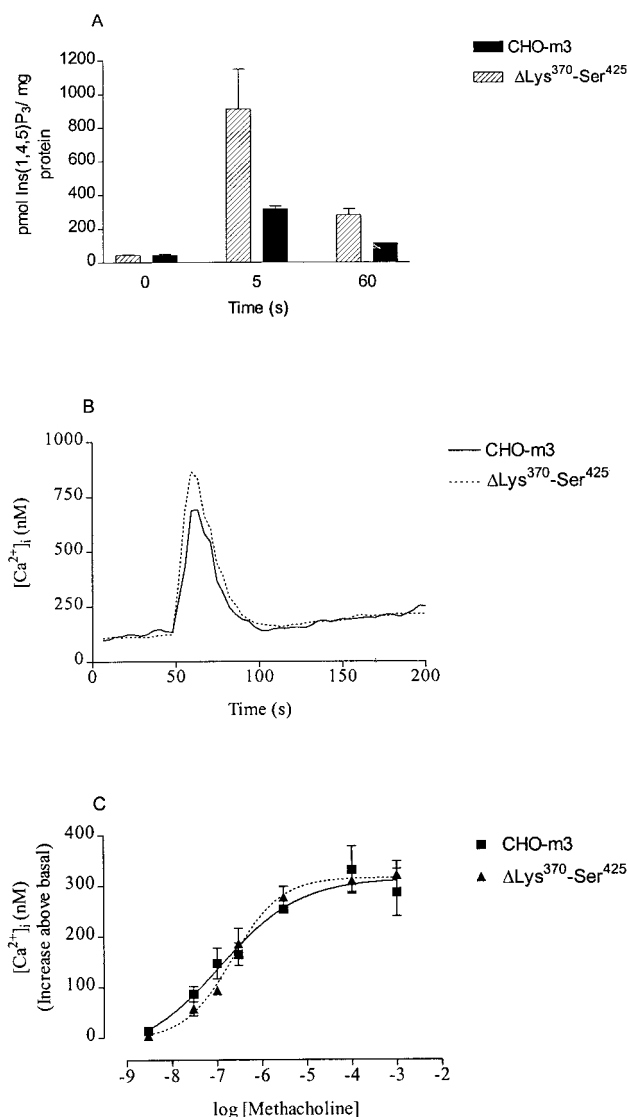


FIG. 4. Coupling of the wild-type M₃-muscarinic receptor and Δ Lys³⁷⁰-Ser⁴²⁵ receptor mutant to the phospholipase C pathway. A, Ins(1,4,5)P₃ generation was determined in cells that had been stimulated with carbachol (1 mM) for varying times. The data represent the mean \pm S.E. of three experiments carried 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 (1 mM). C, concentration-response curve of the peak Ca²⁺ response following stimulation with methacholine. The data represent the mean \pm S.E. of at least three experiments.

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-CK1 α -K46R and the 3i-loop peptide into CHO-m3 cells stably expressing the M₃-muscarinic receptor. Expression of F-CK1 α -K46R and the 3i-loop peptide resulted in the reduction of the carbachol-mediated ERK-1/2 response by $53.9 \pm 7.7\%$ and $49.4 \pm 3.2\%$, respectively (Fig. 5A).

Control experiments were designed to test the ability of F-CK1 α -K46R or the 3i-loop peptide to inhibit nonreceptor-mediated ERK-1/2 activation. Hence, the effect of transient transfection of F-CK1 α -K46R or the 3i-loop peptide on the

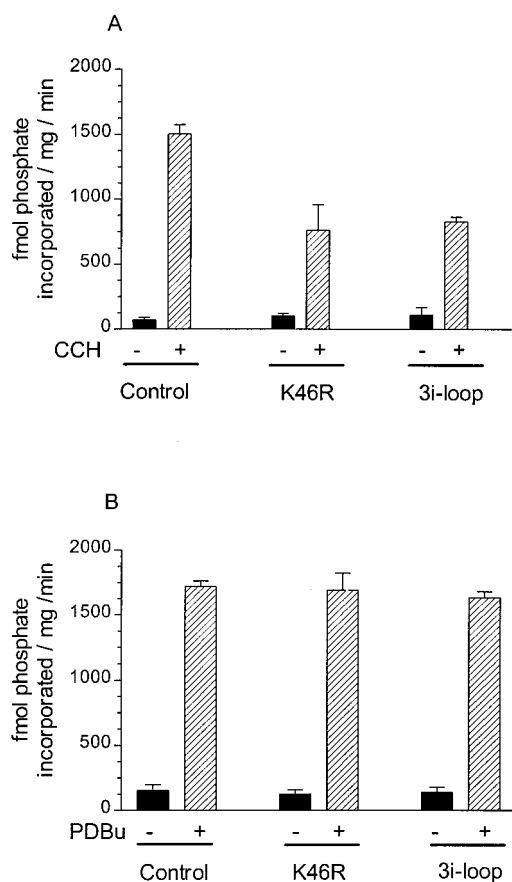


FIG. 5. Effect of the CK1 α dominant negative mutant (F-CK1 α K46R) and the 3i-loop peptide on the M₃-muscarinic ERK-1/2 response. CHO-m3 cells stably expressing recombinant M₃-muscarinic receptors (A) or native CHO-K1 cells (B), were transiently transfected with the CK1 α dominant negative mutant, F-CK1 α K46R (K46R) or the 3i-loop peptide (*3i-loop*) corresponding to Ser³⁴⁵-Leu⁴⁶³ of the third intracellular loop of the M₃-muscarinic receptor or were sham transfected (Control). 48 h after transfection, cells were stimulated with 1 mM carbachol (CCH, A) or 1 μ M phorbol 12,13-dibutyrate (PDBu, B), for 5 min. Reactions were terminated using lysis buffer, and ERK-1/2 activity was determined. The data represent the mean \pm S.E. of three experiments.

phorbol 12,13-dibutyrate ERK-1/2 response in native CHO-K1 cells was tested. It was found that neither F-CK1 α -K46R or the 3i-loop peptide had any significant effect on the phorbol ester-mediated ERK-1/2 response in these cells (Fig. 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-CK1 α -K46R construct inhibited the phorbol ester response by 31% (data not shown). The fact that the F-CK1 α -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 because of 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-CK1 α -K46R significantly ($p < 0.05$, Student's t test) reduced the potency of carbachol by 15.7-fold and 1.8-fold, respectively (Fig. 6).

DISCUSSION

Despite intensive research, the mechanisms employed by GPCRs in the activation of the ERK-1/2 pathway are generally

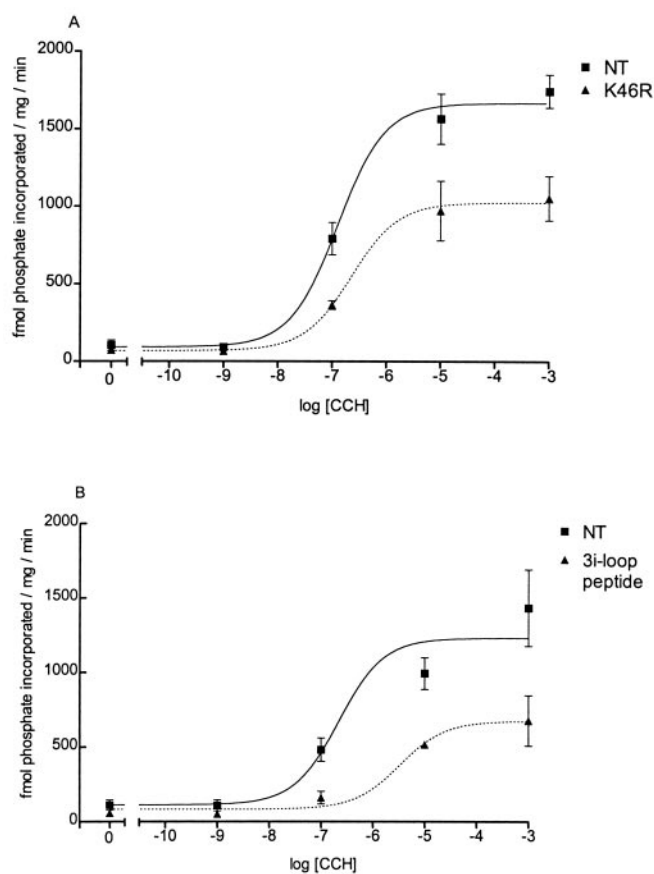


FIG. 6. ERK-1/2 concentration-response curves in CHO-m3 cells transiently transfected with the CK1 α dominant negative mutant (F-CK1 α K46R) and the 3i-loop peptide. CHO-m3 cells stably expressing recombinant M₃-muscarinic receptors were transiently transfected with the CK1 α dominant negative mutant, F-CK1 α K46R (K46R) (A), or the 3i-loop peptide (*3i-loop peptide*, B) corresponding to Ser³⁴⁵-Leu⁴⁶³ of the third intracellular loop of the M₃-muscarinic receptor. 48 h 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 was determined. The data represent the mean \pm S.E. of three experiments.

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 G_{q/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 overriding common feature in the mechanisms employed by G_{q/11}-coupled receptors, namely, the involvement of the G_{q/11}-heterotrimeric G-proteins and the subsequent activation of the phospholipase C signaling pathway. Both the Ins(1,4,5)P₃/Ca²⁺ mobilization and diacylglycerol/PKC arms of the phospholipase C signaling 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 G_{q/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

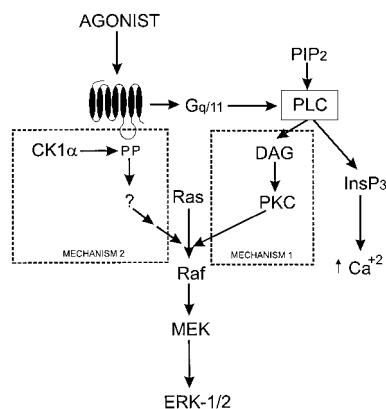


FIG. 7. Scheme of the mechanisms involved in the activation of the ERK-1/2 pathway by M₃-muscarinic receptors. Our data have identified two mechanisms involved in the activation of the ERK-1/2 pathway by M₃-muscarinic receptors expressed in CHO cells. Mechanism 1 is PKC-dependent and is essential in 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 2 (i.e. the $\Delta\text{Lys}^{370}\text{-Ser}^{425}$ receptor mutant or the wild-type M₃-muscarinic receptor expressed together with the 3i-loop peptide or F-CK1 α K46R) will give a less than maximal ERK-1/2 response. CK1 α , casein kinase 1 α ; DAG, diacylglycerol; InsP₃, inositol 1,4,5-trisphosphate; PIP₂, phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C.

PKC by the M₃-muscarinic receptor is sufficient to stimulate ERK-1/2. This conclusion could be applied to a large number of G_{q/11}-coupled receptors that show PKC-dependent activation of ERK-1/2, such as prostaglandin F_{2 α} (15), P_{2Y2}-purinergic (20, 29), CCK (18), M₁-muscarinic, α_1 -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 G_{q/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 $\Delta\text{Lys}^{370}\text{-Ser}^{425}$ M₃-muscarinic receptor mutant would suggest that this simple model is not correct. Deletion of Lys³⁷⁰-Ser⁴²⁵ in the third intracellular loop of the human M₃-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 signaling pathway. In fact this study, consistent with our previous report (35), demonstrates that the $\Delta\text{Lys}^{370}\text{-Ser}^{425}$ 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 signaling pathway was not in itself sufficient to drive a full G_{q/11}-coupled receptor ERK-1/2 response. It is interesting to note that the ERK-1/2 response mediated by the $\Delta\text{Lys}^{370}\text{-Ser}^{425}$ receptor mutant was still sensitive to PKC inhibition. Thus, the $\Delta\text{Lys}^{370}\text{-Ser}^{425}$ 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_{q/11}-activated phospholipase C signaling. This additional mechanism (Fig. 7, Mechanism 2) appears to act in concert with PKC to elicit a full ERK-1/2 response.

These data, therefore, support a model that identifies two mechanisms in the activation of ERK-1/2 (Fig. 7). Mechanism 1 is PKC-dependent and is absolutely required for ERK-1/2 acti-

vation 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_{q/11}-coupled receptor activation of ERK-1/2 is via the activity of the Ca²⁺-sensitive tyrosine kinase Pyk2 or related kinases (25). Ins(1,4,5)P₃-dependent increases in intracellular Ca²⁺ 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 $\Delta\text{Lys}^{370}\text{-Ser}^{425}$ receptor mutant for two reasons. First, the muscarinic receptor ERK-1/2 response in CHO cells is independent of changes in intracellular Ca²⁺ (22) suggesting that Pyk2 is not involved in the M₃-muscarinic receptor response in these cells. Second, GPCR transactivation of RTKs via Pyk2 is a process that involves Ins(1,4,5)P₃-mediated increases in intracellular Ca²⁺ (26). Because the $\Delta\text{Lys}^{370}\text{-Ser}^{425}$ receptor couples efficiently to the phospholipase C pathway, stimulating Ca²⁺ mobilization 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 G_{q/11}/phospholipase C pathway and dispels the notion that G_{q/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 CK1 α 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 $\Delta\text{Lys}^{370}\text{-Ser}^{425}$ receptor mutant, which lacked eight potential serine phospho-acceptor sites and the putative CK1 α binding site (His³⁷⁴-Val³⁹¹) (35). Consistent with our hypothesis, the $\Delta\text{Lys}^{370}\text{-Ser}^{425}$ receptor mutant was reduced in its ability to undergo agonist-dependent phosphorylation by ~80% (35).

The reduced ability of the $\Delta\text{Lys}^{370}\text{-Ser}^{425}$ 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 CK1 α -mediated M₃-muscarinic receptor phosphorylation could be achieved using either a dominant negative mutant of CK1 α , F-CK1 α -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 CK1 α (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 because 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-CK1 α -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 Δ Lys³⁷⁰-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 β_2 -adrenergic receptor to the regulation of ERK-1/2 activity. PKA-mediated phosphorylation of the β_2 -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 G_i-protein $\beta\gamma$ -subunits (31). Furthermore, agonist-mediated GRK-2 phosphorylation has been shown to recruit a β -arrestin-c-Src complex to the β_2 -adrenergic receptor (32). Preventing the ability of β -arrestin to interact with c-Src inhibits β_2 -adrenergic receptor-mediated ERK-1/2 activation, suggesting that recruitment of c-Src to the phosphorylated β_2 -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 β_2 -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 CK1 α initiates a process that acts in concert with PKC to mediate a full M₃-muscarinic receptor ERK-1/2 response (Fig. 7). The exact nature of the mechanism initiated by receptor phosphorylation is presently unclear but appears not to involve G_{q/11} heterotrimeric G-proteins nor the activation of the phospholipase C second messenger signaling 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-c-Src and the β_2 -adrenergic receptor.

Acknowledgments—We thank Prof. Nahorski whom, together with Drs. A. B. Tobin and G. B. Willars, initiated the work on the Δ Lys³⁷⁰-Ser⁴²⁵ receptor mutant.

REFERENCES

- Malarkey, J., Belham, C. M., Paul, A., Graham, A., Scott, P. H., and Plevin, R. (1995) *Biochem. J.* **309**, 361–375
- Wan, Y., Kurosaki, T., and Huang, X.-Y. (1996) *Nature* **380**, 541–544
- Hawes, B. E., van Biesen, T., Koch, W. J., Luttrell, L. M., and Lefkowitz, R. J. (1995) *J. Biol. Chem.* **270**, 17148–17153
- Gutkind, J. S. (1998) *J. Biol. Chem.* **273**, 1839–1842
- Winitz, S., Russell, M., Qian, N.-X., Gardner, A., Dwyers, L., and Johnson, G. L. (1993) *J. Biol. Chem.* **268**, 19196–19199
- Crespo, P., Xu, N., Simmonds, W. F., and Gutkind, J. S. (1994) *Nature* **369**, 418–420
- Koch, W. J., Hawes, B. E., Allen, L. F., and Lefkowitz, R. J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 12706–12710
- Lopez-Illasaca, M., Crespo, P., Pellici, P. G., Gutkind, J. S., and Wetzker, R. (1997) *Science* **275**, 394–397
- Luttrell, L. M., Hawes, B. E., van Biesen, T., Luttrell, D. K., Lansing, T. J., and Lefkowitz, R. J. (1996) *J. Biol. Chem.* **271**, 19443–19450
- Della Rocca, G. J., van Biesen, T., Daaka, Y., Luttrell, D. K., Luttrell, L. M., and Lefkowitz, R. J. (1997) *J. Biol. Chem.* **272**, 19125–19132
- Della Rocca, G. J., Maudsley, S., Daaka, Y., Lefkowitz, R. J., and Luttrell, L. M. (1999) *J. Biol. Chem.* **274**, 13978–13984
- Faure, M., Voyno-Yasenetskaya, T. A., and Bourne, H. R. (1994) *J. Biol. Chem.* **269**, 7851–7854
- Palomero, T., Barros, F., Del Camino, D., Vilorio, C. G., and De La Pena, P. (1998) *Mol. Pharmacol.* **53**, 613–622
- Launay, J.-M., Birraux, G., Bondoux, D., Callebort, J., Choi, D.-S., Loric, S., and Maroteaux, L. (1996) *J. Biol. Chem.* **271**, 3141–3147
- Watanabe, T., Waga, I., Honda, Z.-i., Kurokawa, K., and Shimizu, T. (1995) *J. Biol. Chem.* **270**, 8984–8990
- Zou, Y., Komuro, I., Aikawa, R., Kudo, S., Shiojima, I., Hiroi, Y., Mizuno, T., and Yazaki, Y. (1996) *J. Biol. Chem.* **271**, 33592–33597
- Velarde, V., Ullian, M. E., Morinelli, T. A., Mayfield, R. K., and Jaffa, A. A. (1999) *Am. J. Physiol.* **277**, C253–C261
- Tapia, J. A., Ferris, H. A., Jensen, R. T., and Garcia, L. J. (1999) *J. Biol. Chem.* **274**, 31261–31271
- Venkatakrishnam, G., Salgia, R., and Groopman, J. E. (2000) *J. Biol. Chem.* **275**, 6868–6875
- Soltoff, S. P., Avraham, H., Avraham, S., and Cantley, L. C. (1998) *J. Biol. Chem.* **273**, 2653–2660
- Budd, D. C., Rae, A., and Tobin, A. B. (1999) *J. Biol. Chem.* **274**, 12355–12360
- Wylie, P. G., Challiss, R. A. J., and Blank, J. L. (1999) *Biochem. J.* **338**, 619–628
- Kim, J.-Y., Yang, M.-S., Oh, C.-D., Kim, K.-T., Kang, S.-S., and Chun, J.-S. (1999) *Biochem. J.* **337**, 275–280
- Slack, B. E. (2000) *Biochem. J.* **348**, 381–387
- Dikic, I., Tokiwa, G., Lev, S., Courtneidge, S. A., and Schlessinger, J. (1996) *Nature* **383**, 547–550
- Lev, S., Moreno, H., Martinez, R., Canoll, P., Peles, E., Musacchio, J. M., Plowman, G. D., Rudy, B., and Schlessinger, J. (1995) *Nature* **376**, 737–745
- Eguchi, S., Numaguchi, K., Iwasaki, H., Matsumoto, T., Yamakawa, T., Utsunomiya, H., Motley, E. D., Kawakatsu, H., Owada, K. M., Hirata, Y., Marumo, F., and Inagami, T. (1998) *J. Biol. Chem.* **273**, 8890–8896
- Zwick E., Daub, H., Aoki, N., Yamaguchi-Aoki, Y., Tinhofer, I., Maly, K., and Ullrich, A. (1997) *J. Biol. Chem.* **272**, 24767–24770
- Soltoff, S. P. (1998) *J. Biol. Chem.* **273**, 23110–23117
- Pitcher, J. A., Freedman, N. J., and Lefkowitz, R. J. (1998) *Annu. Rev. Biochem.* **67**, 653–692
- Daaka, Y., Luttrell, L. M., and Lefkowitz, R. J. (1997) *Nature* **390**, 88–91
- Luttrell, L. M., Ferguson, S. S. G., Daaka, Y., Miller, W. E., Maudsley, S., Della Rocca, G. J., Lin, F.-T., Kawakatsu, H., Owada, K., Luttrell, D. K., Caron, M. G., and Lefkowitz, R. J. (1999) *Science* **283**, 655–660
- Tobin, A. B., and Nahorski, S. R. (1993) *J. Biol. Chem.* **268**, 9817–9823
- Tobin, A. B., Totty, N. F., Sterlin, A. E., and Nahorski, S. R. (1997) *J. Biol. Chem.* **272**, 20844–20849
- Budd, D. C., McDonald, J. E., and Tobin, A. B. (2000) *J. Biol. Chem.* **275**, 19667–19675
- Tobin, A. B., Lambert, D. G., and Nahorski, S. R. (1992) *Mol. Pharmacol.* **42**, 1042–1048
- Challiss, R. A. J., Batty, I. H., and Nahorski, S. R. (1988) *Biochem. Biophys. Res. Commun.* **157**, 684–691
- Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) *J. Biol. Chem.* **260**, 3440–3450
- Xiong, L., Lee, J. W., Graves, L. M., and Earp, H. S. (1998) *EMBO J.* **17**, 2574–2583
- Cobb, M. H., and Goldsmith, E. J. (1995) *J. Biol. Chem.* **270**, 14843–14846
- Tobin, A. B., Keys, B., and Nahorski, S. R. (1996) *J. Biol. Chem.* **271**, 3907–3916
- Waugh, M. G., Challiss, R. A. J., Berstein, G., Nahorski, S. R., and Tobin, A. B. (1999) *J. Biochem.* **338**, 175–183