Phosphorylation and Regulation of a G q/11-coupled Receptor by Casein Kinase 1α*

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Agonist-mediated receptor phosphorylation by one or more of the members of the G-protein receptor kinase (GRK) family is an established model for G-protein-coupled receptor (GPCR) phosphorylation resulting in receptor desensitization. Our recent studies have, however, suggested that an alternative route to GPCR phosphorylation may be an operation involving casein kinase 1α (CK1α). In the current study we investigate the involvement of CK1α in the phosphorylation of the human m3-muscarinic receptor in intact cells. We show that expression of a catalytically inactive mutant of CK1α, designed to act in a dominant negative manner, inhibits agonist-mediated receptor phosphorylation by ~40% in COS-7 and HEK-293 cells. Furthermore, we present evidence that a peptide corresponding to the third intracellular loop of the m3-muscarinic receptor (Ser245-Leu465) is an inhibitor of CK1α due to its ability to both act as a pseudo-substrate for CK1α and form a high affinity complex with CK1α. Expression of this peptide was able to reduce both basal and agonist-mediated m3-muscarinic receptor phosphorylation in intact cells. These results support the notion that CK1α is able to mediate GPCR phosphorylation in an agonist-dependent manner and that this may provide a novel mechanism for GPCR phosphorylation. The functional role of phosphorylation was investigated using a mutant of the m3-muscarinic receptor that showed an ~80% reduction in agonist-mediated phosphorylation. Surprisingly, this mutant underwent agonist-mediated desensitization suggesting that, unlike many GPCRs, desensitization of the m3-muscarinic receptor is not mediated by receptor phosphorylation. The inositol (1,4,5)-trisphosphate response did, however, appear to be dramatically potentiated in the phosphorylation-deficient mutant indicating that phosphorylation may instead control the magnitude of the initial inositol phosphate response.

It is now well established that G-protein-coupled receptor (GPCR) phosphorylation is a general phenomenon that controls specific key signaling properties of receptors. Originally associated with receptor desensitization (1, 2), GPCR phosphorylation has now been implicated in a number of processes including receptor internalization (3–6) and as a molecular switch that determines coupling to specific signaling pathways (7, 8). The receptor-specific kinases involved are generally considered to belong to the G-protein-coupled receptor kinase (GRK) family which are characterized by their sequence homology to rhodopsin kinase (GRK-1) and that include the extensively studied β-adrenergic receptor kinases 1 and 2 (GRK-2 and -3, respectively) (2, 9). Reconstitution experiments using purified, or partially purified, receptors have demonstrated that in addition to the β 2-adrenergic receptor a number of GPCRs including muscarinic ((10–12), substance P (13), bradykinin B 2 (14), and adenosine A3 receptors (15) can act as GRK substrates. Furthermore, a GRK-2 dominant negative mutant (16) has been widely employed to probe the role of endogenous GRK-2 in the regulation of GPCRs (3, 17–19). These studies, and others, have led to the proposal that the GRKs, and in particular GRK-2, have a broad receptor substrate specificity and are able to phosphorylate and regulate GPCRs coupled to both adenyl cyclase via G α s and those coupled to the phospholipase C pathway via G α 11.

In contrast to this model of GRK-mediated phosphorylation of GPCRs, our studies on the G q/11-coupled m3-muscarinic receptor have suggested that there may be an alternative mechanism mediating agonist-dependent receptor phosphorylation. This receptor is rapidly phosphorylated on serine following agonist addition (20) with a time course that closely correlates with receptor desensitization as measured by diminished inositol (1,4,5)-trisphosphate (Ins(1,4,5) P3) and intracellular calcium responses (21). Initial characterization of the kinase involved in this phosphorylation event eliminated a role for protein kinase A, protein kinase C, and Ca2+/calmodulin-dependent protein kinase (20). Crude membranes prepared from CHO cells expressing recombinant m3-muscarinic receptor were also found to contain receptor kinase activity and that this activity was insensitive to inhibition by heparin and zinc at concentrations that were known to inhibit GRK-2 activity (22). These were the first data suggesting that the m3-muscarinic receptor was phosphorylated by a kinase that was distinct from GRK-2. By using a bacterial fusion protein of the third intracellular loop of the m3-muscarinic receptor as a pseudo-substrate for the “putative” muscarinic receptor kinase, we were able to purify, from porcine cerebellum, a 40-kDa protein kinase that in membrane reconstitution experiments was able to phosphorylate the m3-muscarinic receptor in an agonist-dependent manner (23). Amino acid sequence analysis identified this protein kinase as casein kinase 1α (CK1α) (24). Importantly, the ability of CK1α to drive receptor phosphorylation was not restricted to the m3-muscarinic receptor since both rhodopsin and the m1-muscarinic receptor were also shown to be in vitro substrates that were phosphorylated in a stimulus-dependent manner (24, 25). These in vitro studies suggested

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The abbreviations used are: GPCR, G-protein-coupled receptor; CK1α, casein kinase 1α; GRK, G-protein-coupled receptor kinase; Ins(1,4,5) P3, inositol (1,4,5)-trisphosphate; CHO, Chinese hamster ovary; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase.

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that CK1α may act as a cellular kinase for specific GPCRs, thereby offering an alternative and distinct route to GPCR phosphorylation from that of the GRKs.

In the present study we explore this hypothesis further by using a catalytically inactive mutant of CK1α and a peptide corresponding to the third intracellular loop of the m3-muscarinic receptor to inhibit endogenous CK1α activity. These experiments provide evidence for a cellular role of CK1α in the phosphorylation and regulation of the m3-muscarinic receptor.

**MATERIALS AND METHODS**

*Cell Culture—* COS-7, HEK-293, and CHO cells were grown in medium containing α-minimum Eagle’s medium 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.

*Generation of F-CK1αK46R—* Wild-type bovine casein kinase 1α that had been tagged at the N terminus with the FLAG epitope (F-CK1α) and cloned into pcDNA-3 (Invitrogen, see Ref. 24) was used as a template for the Quikchange site-directed mutagenesis kit (Stratagene). The mutagenesis primer used was GAGGAGTGCGAAGCTGCACTAAGATCCCAAGGCGGAGGCTCAGGTTG. This created a Lys-Arg change at position 46 in the amino acid sequence of CK1α. The resulting construct F-CK1αK46R was contained in pcDNA-3 and also possessed a FLAG epitope tag on the N terminus. The mutation was confirmed by DNA sequencing.

*Generation of the Third Intracellular Loop Peptide (3i Loop Peptide)—* The sequence encoding amino acids Ser456-Leu463 in the third intracellular loop of the m3-muscarinic receptor was amplified using the m3-muscarinic chain reaction primers, 5’ primer, GGGGCTACCGCCACACATGTCCCTGGAGAACTCCGCCTCCTCCGAC, and 3’ primer, GGGTCTGACTACAGATGGTGGCTCTGAGGACAGAGG, and cloned into KpnI and XbaI sites in pcDNA-3. The resulting construct was then used in transient transfections of HEK-293 cells or COS-7 cells or used to make stably expressing CHO cell lines.

*GST-M3-Muscarinic Receptor Deletion Mutants—* Lys770, Ser825 Deletion Mutant—The m3-muscarinic receptor coding sequence contained in pcDNA-3 was digested with HindIII and then religated. This removed the coding sequence for amino acids Lys770-Ser825 inclusive but maintained the reading frame of the remaining cDNA.

*Construction of Bacterial Fusion Proteins—* Generation of the GST bacterial fusion proteins used in this study have been described previously (23). Antibody Production—Production and characterization of the m3-muscarinic receptor specific antisera raised against residues Ser465-Leu463 in the third intracellular loop of the m3-muscarinic receptor has been previously described (20). The pan-M antiserum was raised against a peptide (DRYSFSTRLYSRKRTPRC) corresponding to amino acids Asp122–Arg140 of the m1-muscarinic receptor. This sequence is conserved in the muscarinic receptor family, and the resulting antisera would be expected to cross-react with all of the muscarinic receptor subtypes. The peptide was conjugated to Keyhole Limpet hemocyanin and injected into New Zealand White rabbits using standard protocols. Characterization of the antisera using Western blots showed that the pan-M antiserum cross-reacted with the m1 and m3 muscarinic receptors. This antibody was used in immunoprecipitations where the phosphorylation of the Lys770-Ser825 deletion mutant was investigated.

The CK1α-specific antisera was raised against a peptide corresponding to the N terminus of bovine CK1α (MASSGSQAEFFVGGKYKLC). Characterization of the antisera using Western blots showed that it was able to cross-react with purified recombinant bovine CK1α and endogenous CK1α present in CHO, HEK-293, COS-7 cells, and rat brain.

*Transient Transfections of HEK-293, CHO, and COS-7 Cells—* Cells were plated onto 12-well dishes 24 h before transfection (cells were 40–60% confluent at the start of transfection). Cells were transfected with m3-muscarinic receptor cDNA (contained in pcDNA-3) or co-transfected with m3-muscarinic receptor plus F-CK1αK46R or 3i loop peptide constructs. The transfection reagent used was Fugene (Roche Molecular Biochemicals) using a total DNA concentration of 0.5 μg/well. In experiments to determine inositol (1,4,5)-triphosphate levels, HEK-293 cells were plated onto 24-well dishes, and each well was transfected with 0.25 μg of DNA. Cells were used 48–72 h after transfection.

*Stable Transfection Of CHO Cells with the Third Intracellular Loop Peptide (3i Loop Peptide)—* CHO-K1 cells were transiently transfected with the 3i loop peptide construct (described above) using the Fugene method. Clones were screened for expression by Western blot using the m3-muscarinic receptor antisera that was raised against this peptide (20). The resulting cells were then used in experiments where the m3-muscarinic receptor was transiently transfected.

*Immunoprecipitation of Phosphorylated m3-Muscarinic Receptor and Third Intracellular Loop Peptide—* Cells plated onto 12-well dishes were washed in phosphate-free Krebs/HEPES buffer (10 mM HEPES, 118 mM NaCl, 4.3 mM KCl, 1.17 mM MgSO₄·7H₂O, 1.35 mM CaCl₂, 25.0 mM NaHCO₃, 7.4 mM glucose, 0.4 mM NaH₂PO₄, pH 7.4) and incubated in phosphate-free Krebs/HEPES supplemented with [³²P]orthophosphate (50 μCi/ml) for 1–2 h at 37 °C. Either vehicle or the cholinergic agonist, carbachol (0.1 mM), was added, and incubations were continued for a further 5 min. Reactions were terminated by rapid aspiration of the drug-containing media and application of 1 ml of ice-cold solubilization buffer (10 mM Tris, 10 mM EDTA, 500 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, pH 7.4). Samples were left on ice for 15 min and then cleared by microcentrifugation. Antiserum (0.2 μg/ml) was added, and the samples were left on ice for 60–90 min. Immune complexes were isolated on protein A-Sepharose beads, and the beads were washed three times with TE buffer (10 mM Tris·HCl, 10 mM EDTA, pH 7.4). In the case of receptor immunoprecipitations, the protein pellet was then resuspended in 1 ml PBS and aliquots of the protein A slurry resuspended corresponding to a known quantity of receptors determined by radioreceptor assay (see below). This ensured that for each experiment the same number of receptors from each transfection was run on the gel. Isolated immune complexes were then resolved on 8% SDS-PAGE gels in the case of muscarinic receptors or 15% gels for the 3i loop peptide. The gels were dried and subjected to autoradiography, and the level of phosphorylation was assessed with a Bio-Rad model GS 670 densitometer.

*Quantification of m3-Muscarinic Receptor Expression—* m3-Muscarinic receptor expression for each transfection was determined by incubating cells plated down onto 12-well dishes with 0.5 ml of Krebs/HEPES buffer (as above but containing KH₂PO₄·1.17 mM) containing a saturating concentration of muscarinic receptor antagonist [³²H]-N-methylscopolamine (1 nM) (26). Extracts were brought to pH 7 by addition of NaH₂CO₃, and bound [³²H]-N-methylscopolamine was determined by liquid scintillation counting of cell extracts solubilized in solubilization buffer. Non Specific binding was determined in the presence of 10 μM atropine and was <3% of the total binding.

*GST Fusion Protein Pull Down Assay—* The hippocampus and cerebral cortex from one rat was homogenized in 15 ml of TE buffer (10 mM Tris·HCl, 2.5 mM EDTA, pH 7.4) for 1 h at 4 °C. GST fusion protein complexes were isolated on glutathione-Sepharose beads and washed three times in TE buffer. beads were resuspended in Laemmli buffer and resolved by 12% SDS-PAGE. The presence of CK1α was then detected using Western blot using the casein kinase 1α-specific antibody.

*In Vitro Kinase Assay for FLAG-tagged Casein Kinase 1α (F-CK1α) and F-CK1αK46R—* HEK-293 cells were transiently transfected with either recombinant bovine FLAG-tagged F-CK1α, FLAG-tagged F-CK1αK46R, or vehicle. 72 h after transfection cells were lysed with 1 ml ice-cold lysis buffer (20 mM HEPES, 10 mM EDTA, 0.5% Nonidet P-40, 250 mM NaCl, 3% EDTA, 3% EGTA, 2% NaVO₃, 1% diethioleitol, 1% phenylmethylsulfonyl fluoride, pH 7.4). The lysate was centrifuged at 21,000 × g for 5 min at 4 °C and the pellet discarded. The lysate was added mouse M2 anti-FLAG antibody (0.1 μg) for 1 h at 4 °C followed by rabbit anti-mouse IgG (1 μg) for 20 min at 4 °C. The immune complexes isolated on protein A-Sepharose beads and washed twice with lysis buffer and twice with kinase buffer (10 mM Tris·HCl, 1
RESULTS

Generation of a Catalytically Inactive Mutant of CK1α—To investigate whether the cellular kinase responsible for m3-muscarinic receptor phosphorylation was CK1α, we tested the ability of a catalytically inactive form of CK1α to inhibit agonist-mediated m3-muscarinic receptor phosphorylation. Lysine 46 in bovine CK1α corresponds to the conserved lysine found at the ATP-binding site of all protein kinases (27). By point mutagenesis we constructed a lysine to arginine mutation at position 46 (called F-CK1αK46R) that would be predicted to result in a catalytically inactive kinase. Expression of F-CK1αK46R was confirmed in transiently transfected HEK-293 cells by Western blotting for the FLAG epitope that was engineered at the N terminus (Fig. 1A). Due to the epitope tag the recombinant mutant ran at a slightly higher molecular mass than the endogenous CK1α. Hence by Western blotting with a polyclonal CK1α antisera that detected both endogenous CK1α and recombinant mutant kinase, we estimated that F-CK1αK46R and endogenous CK1α were expressed at approximately equivalent levels (Fig. 1B). Similar results were obtained for F-CK1αK46R expressed in COS-7 cells (data not shown).

In order to determine enzymatic activity, HEK-293 cells were transiently transfected with recombinant bovine F-CK1α or F-CK1αK46R, both of which were tagged at the N terminus with the FLAG epitope. In vitro kinase assays on FLAG antisera immunoprecipitates revealed that the F-CK1αK46R had no detectable kinase activity (Fig. 1C).

F-CK1αK46R Decreases Agonist-mediated Receptor Phosphorylation in Intact Cells—Human m3-muscarinic receptors transfected into HEK-293 or COS-7 cells were phosphorylated in an agonist-dependent manner by endogenous protein kinase(s) (Fig. 2). Co-transfection of the m3-muscarinic receptor with F-CK1αK46R resulted in a decrease in agonist-mediated receptor phosphorylation by 40.1 ± 2.0% (n = 3, ±S.E.) and 43.1 ± 3.5% (n = 3, ±S.E.) in HEK-293 cells and COS-7 cells, respectively (Fig. 2). In these experiments cells were stimulated with a maximum concentration of agonist (carbachol; 100 μM) for 5 min, conditions that we have previously reported results in maximum phosphorylation of the receptor (20).

In each experiment receptor expression was determined, and the amount of receptor applied to the gel was adjusted so that equal receptor numbers were run on the gel. Co-expression of the F-CK1αK46R with the receptor did not influence the level of m3-muscarinic receptor expression. Any differences we observed in the level of receptor expression between control and co-transfected cells (usually <30%) were probably due to experimental variations in transfection efficiencies.

A Peptide Corresponding to the Third Intracellular Loop of the m3-Muscarinic Receptor Inhibits Receptor Phosphorylation—Earlier studies from our laboratory demonstrated that a bacterial fusion protein containing a portion of the third intracellular loop of the m3-muscarinic receptor (Ser345–Leu463) was able to inhibit CK1α-mediated muscarinic receptor phosphorylation in membranes (Ref. 23; also see "Discussion"). Here we tested the ability of this peptide to inhibit m3-muscarinic receptor phosphorylation in intact cells.

Expression of the transfected peptide, of ∼12.5 kDa, corresponding to amino acids Ser345–Leu463 (3i loop peptide) of the m3-muscarinic receptor was detected by Western blotting using an m3-muscarinic receptor antiserum that was raised against this peptide (20) (Fig. 3). Transient co-expression of m3-muscarinic receptors with the 3i loop peptide in COS-7 cells resulted in a decrease in agonist-mediated m3-muscarinic receptor phosphorylation by 72.0 ± 5.9% (n = 3, ±S.E.) (Fig. 4). Interestingly, the basal phosphorylation seen in COS-7 cells was also reduced (∼60%) by the 3i loop peptide (Fig. 4). In HEK-293 cells m3-muscarinic receptor phosphorylation was also inhibited by expression of the 3i loop peptide, in this case by 45.9 ± 2.9% (n = 3, ±S.E.) (Fig. 4).

We also developed a stable CHO cell line that expressed the 3i loop peptide constitutively. This cell line was transiently transfected with the m3-muscarinic receptor and receptor phosphorylation compared with native CHO-K1 cells transiently transfected with the receptor. In these experiments...
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expression of the 3i loop peptide reduced agonist-mediated phosphorylation of the m3-muscarinic receptor by 75.2 ± 3.4% (n = 4, ±S.E.). Furthermore, basal phosphorylation was also reduced in the presence of the 3i loop peptide (by ~50%).

The 3i loop peptide expressed in CHO cells was itself phosphorylated, but this phosphorylation was not altered by m3-muscarinic receptor stimulation (Fig. 5). The same results were obtained in COS-7 and HEK-293 cells (data not shown).

Determination of a Putative CK1α-binding Site on the m3-Muscarinic Receptor—CK1α contained in a crude soluble rat brain fraction specifically associated with the muscarinic receptor portion of a glutathione S-transferase (GST) bacterial fusion protein that contains the third intracellular loop sequence Ser345-Leu463, designated Ex-m3 (Fig. 6). This interaction appeared particularly strong since washes in salt (KCl) up to a concentration of 2 M was not sufficient to disrupt binding of CK1α (data not shown). Deletion mutants of Ex-m3 were used to map the binding site of CK1α (Fig. 7A). Truncation at the N- and C-terminals of Ex-m3 did not affect the ability of the fusion protein to interact with CK1α present in rat brain supernatant (Fig. 7B) or recombinant bovine CK1α partially purified from infected s f-9 cells (data not shown). However, deletion of the region Lys370–Ser425 (ΔLys370–Ser425) resulted in no detectable binding of CK1α (Fig. 7B). A smaller deletion of 18 amino acids (His374–Val391) also resulted in a fusion protein that was unable to associate with CK1α (Fig. 7B). (Note, the identity of the doublet in the Ex-m3 pull down (lane 2, Fig. 7C) is likely to be CK1α running at its correct molecular mass (the lower band) and CK1α that is still associated with Ex-m3 (upper band). The doublet in lane 5 (Fig. 7C) is likely to be CK1α (the upper band) and an unknown protein that cross-reacts with the CK1α antiserum (lower band.).

Deletion of the Region Lys370–Ser425 from the Third Intracellular Loop of the m3-Muscarinic Receptor Results in Reduced Agonist-mediated Phosphorylation—Our previous studies have demonstrated that CK1α-mediated phosphorylation of the bacterial fusion protein ΔLys370–Ser425 is reduced from that of the full-length fusion protein, Ex-m3 (23). It was, therefore, decided to make the same deletion in the intact m3-muscarinic receptor with the aim to produce a receptor unable to undergo agonist-mediated phosphorylation.

Hence a stable CHO cell line was generated expressing a m3-muscarinic receptor containing a Lys370–Ser425 deletion in the third intracellular loop. The clone used (clone 17) was carefully selected to have a similar receptor expression level as our previous CHO cell line expressing the wild type m3-muscarinic receptor (m3-R) in the 12.5-kDa 3i loop peptide. The results shown are representative of three experiments. The position of molecular mass markers are shown in kilodaltons.

![Figure 2](http://www.jbc.org/)

**Fig. 2.** Expression of F-CK1αK46R reduces agonist-mediated phosphorylation of the m3-muscarinic receptor. Cells expressing the m3-muscarinic receptor alone (CNT) or co-transfected with F-CK1αK46R (K46R) were prelabeled with [32P]orthophosphate and stimulated with 0.1 mM carbachol (CCh) or recombinant bovine CK1α (Fig. 7A). A, a representative gel from an experiment using transiently transfected HEK-293 cells. B, a representative gel from an experiment using transiently transfected COS-7 cells. In this example m3-muscarinic receptor levels were ~1.2 pmol/mg protein. A, representative gel from an experiment using transiently transfected HEK-293 cells. In this example m3-muscarinic receptor levels were ~0.8 pmol/mg protein. The data shown are representative of at least three experiments. The positions of molecular mass markers are shown in kilodaltons.

![Figure 3](http://www.jbc.org/)

**Fig. 3.** Expression of the third intracellular loop peptide Ser345-Leu463 (3i loop peptide). A, diagrammatic representation showing the region in the m3-muscarinic receptor that corresponds to the 12.5-kDa 3i loop peptide. B, Western blot using the m3-muscarinic receptor antiserum of cell lysates from cells not transfected (NT) or transfected with the intact m3-muscarinic receptor (m3-R) or the 3i loop peptide (3i-P). The results shown are representative of three experiments. The position of molecular mass markers are shown in kilodaltons.

![Diagram](http://www.jbc.org/)

**Diagram:** Diagrammatic representation showing the region in the m3-muscarinic receptor that corresponds to the 12.5-kDa 3i loop peptide.
orthophosphate and stimulated with 0.1 mM carbachol (CCh) were prelabeled with 

$^{32}$P-

Cells expressing the m3-muscarinic receptor alone (agonist-mediated phosphorylation of the m3-muscarinic receptor) were transiently transfected with the m3-muscarinic receptor. Cells were prelabeled with $^{32}$Porthophosphate and stimulated with 0.1 mM carbachol for 5 min. Cells were then lysed with solubilization buffer, and the 3i loop peptide was immunoprecipitated using the m3-muscarinic receptor-specific antiserum. The immunoprecipitate was resolved on a 15% SDS-PAGE gel. The data shown are representative of three experiments. The positions of molecular mass markers are shown in kilodaltons.

Ser$^{425}$ deletion mutant and wild type m3-muscarinic receptor, respectively.

Since the polyclonal m3-muscarinic receptor-specific antiserum used throughout this study was raised against the region Ser$^{425}$-Leu$^{463}$ it was possible that the Lys$^{370}$-Ser$^{425}$ deletion mutant may have epitopes removed that would prevent immunoprecipitation by this antiserum. For this reason an alternative antiserum was raised against a peptide conserved among the muscarinic receptor family (Asp$^{122}$Arg$^{140}$) in the 2nd intracellular loop of the m1-muscarinic receptor. This antiserum recognized both m1- and m3-muscarinic receptors as determined by Western blot (data not shown) and was designated pan-M.

By using the pan-M antiserum in immunoprecipitation studies, it was found that the level of agonist-mediated phosphorylation of the receptor containing the Lys$^{370}$-Ser$^{425}$ deletion was dramatically reduced (~80%) compared with wild type receptor (Fig. 8). Note that the Lys$^{370}$-Ser$^{425}$ deletion mutant runs at ~90 kDa compared with the wild type muscarinic receptor that runs at ~110 kDa.

The pan-M antiserum did, however, cross-react with phosphoproteins other than the m3-muscarinic receptor as evident by bands at ~120 and ~82 kDa in non-transfected control cells (Fig. 8). The identities of these proteins are not known.

Analysis of the Ins(1,4,5)P$_3$ Response in the Lys$^{370}$-Ser$^{425}$ Deletion Mutant of the m3-Muscarinic Receptor—These experiments were carried out on CHO cells stably expressing either the wild type m3-muscarinic receptor or the Lys$^{370}$-Ser$^{425}$ deletion mutant. Analysis of the wild type m3-muscarinic receptor Ins(1,4,5)P$_3$ response to agonist challenge showed a characteristic peak of Ins(1,4,5)P$_3$ production followed by a plateau phase (Fig. 9A) consistent with previous reports (21, 28). Also consistent with previous studies was the demonstration that the peak Ins(1,4,5)P$_3$ response could be desensitized by 41.5% following a 5-min pre-stimulation with agonist (Fig. 9A) (21, 28).

The temporal profile of the Ins(1,4,5)P$_3$ response on agonist stimulation of the Lys$^{370}$-Ser$^{425}$ deletion mutant was similar to the wild type receptor (Fig. 9B). Importantly, despite the fact that agonist-mediated phosphorylation of the Lys$^{370}$-Ser$^{425}$ deletion mutant was dramatically reduced, this receptor was...
deletion mutant and wild type m3-muscarinic receptor (Fig. 9C). The control wild type receptor peak response was 249.7 ± 5.4 pmol of Ins(1,4,5)P3/mg protein compared with 517.3 ± 72.0 pmol of Ins(1,4,5)P3/mg protein (n = 3 ± S.E.) in the case of the Lys370–Ser425 deletion mutant. This increased in Ins(1,4,5)P3 production observed on stimulation of the mutant receptor was restricted to the peak response since the plateau responses for the mutant and wild type receptors were similar (Fig. 9C). Interestingly, despite the greater responsiveness of the Lys370–Ser425 deletion mutant in terms of the magnitude of the Ins(1,4,5)P3 response, there was no significant difference (p > 0.05 Student’s t test) in the potency of the carbachol-mediated Ins(1,4,5)P3 elevation between the wild type receptor and the Lys370–Ser425 deletion mutant (EC50 values for wild type and Lys370–Ser425 deletion mutant receptors were 7.14 ± 3.2 and 9.71 ± 1.9 µM (n = 3, ± S.E.), respectively).

The ability of the Lys370–Ser425 deletion mutant to show increased stimulation of inositol phosphate production was also tested in transiently transfected HEK-293 cells. Such experiments are free from the potential clonal artifacts of the stably transfected cell lines. In these experiments the Lys370–Ser425 deletion mutant peak (10 s) Ins(1,4,5)P3 response was 24.3 ± 0.4% (n = 3) greater than the wild type receptor response (data not shown). The fact that the increased responsiveness of the Lys370–Ser425 deletion mutant was not as great as that observed in the stable transfections may be due to the different experimental protocol and cell lines; however, the trend is the same, namely the Lys370–Ser425 deletion mutant appears to generate a greater Ins(1,4,5)P3 response than the wild type receptor.

Functional Analysis of Transiently Transfected HEK-293 Cells Co-expressing the m3-Muscarinic Receptor and F-CK1αK46R—Peak Ins(1,4,5)P3 responses to agonist stimulation was analyzed in HEK-293 cells transiently transfected with the m3-muscarinic receptor only or co-transfected with F-CK1αK46R. Following a 5-min pre-stimulation with agonist and a 5-min wash period, the Ins(1,4,5)P3 response to stimulation of the m3-muscarinic receptor was desensitized by 28.3% (Fig. 10). In cells co-transfected with the m3-muscarinic receptor and the catalytically inactive kinase F-CK1αK46R, a procedure that reduces agonist-mediated receptor phosphorylation (see above), the peak response was still desensitized (by 33.8%) following agonist pre-stimulation (Fig. 10).

Interestingly, the peak Ins(1,4,5)P3 response in cells co-transfected with the m3-muscarinic receptor and the F-CK1αK46R mutant was larger (by 25%) than the peak response of cells transfected with the m3-muscarinic receptor alone (Fig. 10). It therefore appears that transient transfection

FIG. 7. Determination of the site of interaction between CK1α and the third intracellular loop of the m3-muscarinic receptor. A, diagrammatic representation of the muscarinic receptor portion of the GST fusion proteins showing deleted regions. Also shown in triangles are the positions of the serine residues. B, Coomassie Blue stain of the fusion proteins used in the GST fusion protein pull down assay. C, immunodetection of CK1α isolated from pull down experiments where fusion proteins (5 µg) were incubated with rat brain lysate (50 µg of protein). The data shown are representative of four experiments. The position of molecular mass markers are shown in kilodaltons. Key to lanes, lane CK1α, purified recombinant F-CK1α standard; lane B, no fusion protein added; lane 1, GST; lane 2, Ex-m3; lane 3, ΔLys370–Ser425; lane 4, Ex-345–427; lane 5, Ex-376–463; lane 6, ΔHis376–Val391.

still desensitized by 51.2% following a 5-min agonist pre-stimulation (Fig. 9B).

There was, however, a dramatic difference in the magnitude of the peak Ins(1,4,5)P3 responses between the Lys370–Ser425 deletion mutant and wild type m3-muscarinic receptor (Fig. 9C). The control wild type receptor peak response was 249.7 ± 5.4 pmol of Ins(1,4,5)P3/mg protein compared with 517.3 ± 72.0 pmol of Ins(1,4,5)P3/mg protein (n = 3 ± S.E.) in the case of the Lys370–Ser425 deletion mutant. This increased in Ins(1,4,5)P3 production observed on stimulation of the mutant receptor was restricted to the peak response since the plateau responses for the mutant and wild type receptors were similar (Fig. 9C). Interestingly, despite the greater responsiveness of the Lys370–Ser425 deletion mutant in terms of the magnitude of the Ins(1,4,5)P3 response, there was no significant difference (p > 0.05 Student’s t test) in the potency of the carbachol-mediated Ins(1,4,5)P3 elevation between the wild type receptor and the Lys370–Ser425 deletion mutant (EC50 values for wild type and Lys370–Ser425 deletion mutant receptors were 7.14 ± 3.2 and 9.71 ± 1.9 µM (n = 3, ± S.E.), respectively).

The ability of the Lys370–Ser425 deletion mutant to show increased stimulation of inositol phosphate production was also tested in transiently transfected HEK-293 cells. Such experiments are free from the potential clonal artifacts of the stably transfected cell lines. In these experiments the Lys370–Ser425 deletion mutant peak (10 s) Ins(1,4,5)P3 response was 24.3 ± 0.4% (n = 3) greater than the wild type receptor response (data not shown). The fact that the increased responsiveness of the Lys370–Ser425 deletion mutant was not as great as that observed in the stable transfections may be due to the different experimental protocol and cell lines; however, the trend is the same, namely the Lys370–Ser425 deletion mutant appears to generate a greater Ins(1,4,5)P3 response than the wild type receptor.

Functional Analysis of Transiently Transfected HEK-293 Cells Co-expressing the m3-Muscarinic Receptor and F-CK1αK46R—Peak Ins(1,4,5)P3 responses to agonist stimulation was analyzed in HEK-293 cells transiently transfected with the m3-muscarinic receptor only or co-transfected with F-CK1αK46R. Following a 5-min pre-stimulation with agonist and a 5-min wash period, the Ins(1,4,5)P3 response to stimulation of the m3-muscarinic receptor was desensitized by 28.3% (Fig. 10). In cells co-transfected with the m3-muscarinic receptor and the catalytically inactive kinase F-CK1αK46R, a procedure that reduces agonist-mediated receptor phosphorylation (see above), the peak response was still desensitized (by 33.8%) following agonist pre-stimulation (Fig. 10).

Interestingly, the peak Ins(1,4,5)P3 response in cells co-transfected with the m3-muscarinic receptor and the F-CK1αK46R mutant was larger (by 25%) than the peak response of cells transfected with the m3-muscarinic receptor alone (Fig. 10). It therefore appears that transient transfection...
of F-CK1αK46R resulting in decreased receptor phosphorylation has no effect on the ability of the inositol phosphate response to be desensitized but does result in an increase the magnitude of the peak Ins(1,4,5)P₃ response. This represents a radical departure from the widely accepted model for GPCR phosphorylation and desensitization mediated by the GRKs.

Previous in vitro studies from our laboratory have established that CK1α is able to mediate stimulus-dependent phosphorylation of a number of GPCRs (24, 25) suggesting that CK1α may represent a pathway, distinct from the GRKs, for receptor phosphorylation in intact cells. In the present study we have taken advantage of the fact that, like many GPCRs, the m₃-muscarinic receptor is phosphorylated in an agonist-dependent manner by endogenous receptor kinases expressed in a number of commonly used cell lines (i.e. CHO, COS-7, and HEK-293 cells). To investigate whether CK1α was one of these endogenous receptor kinases, we tested the ability of a catalytically inactive mutant of CK1α to inhibit m₃-muscarinic receptor phosphorylation. By mutating a conserved lysine residue (Lys⁴⁶) to an arginine (F-CK1αK46R) in sub-domain II of the catalytic domain of CK1α, known to be essential for phosphorylation (27), the catalytic activity of the kinase was lost. Analogous mutations in other protein kinases, in particular GRK-2, have been used to generate dominant negative mutants (16). Furthermore, mutation of this conserved lysine in CK1ε has been shown to result in a dominant negative mutant able to block endogenous CKI-mediated phosphorylation of dishevelled in Xenopus oocytes (29). We predicted, therefore, that if endogenous CK1α was responsible for m₃-muscarinic receptor phosphorylation then expression of the F-CK1αK46R mutant would inhibit receptor phosphorylation by acting in a dominant negative manner. We show here that F-CK1αK46R when expressed in cells at approximately equivalent levels to the endogenous CK1α resulted in a dramatic reduction in the level of agonist-mediated m₃-muscarinic receptor phosphorylation, suggesting that at least a proportion of the receptor phosphorylation was mediated by endogenous CK1α.

We aimed to confirm this finding by raising an alternative inhibitor to CK1α. Previous studies have shown that a peptide corresponding to the third intracellular loop of the m₃-muscarinic receptor (Ser⁴⁴⁵–Leu⁴⁶⁸) contained in a GST bacterial fusion protein was able to inhibit m₃-muscarinic receptor phosphorylation in membranes (23). This inhibitory property was attributed to the ability of the peptides to act as a pseudo-substrate for CK1α and therefore compete with the receptor for endogenous CK1α present in the membrane preparation. Additionally, in the present study we demonstrate that this peptide forms a high affinity complex with CK1α, and this may also contribute to its inhibitory properties. In order to investi-
gate the involvement of CK1α in the phosphorylation of m3-muscarinic receptors in intact cells, we tested the ability of the third intracellular loop peptide Ser345-Leu463 (3i loop peptide) to inhibit receptor phosphorylation in transfected cell lines. Expression of the 3i loop peptide resulted in a dramatic reduction in both basal and agonist-mediated m3-muscarinic receptor phosphorylation. The peptide itself became phosphorylated suggesting that, as in the membrane experiments, the peptide was acting as a pseudo-substrate for CK1α. Although it is possible that the 3i loop peptide may be inhibiting receptor phosphorylation by interacting with kinases other than CK1α, the cellular data presented here is consistent with the earlier in vitro data and suggests that m3-muscarinic receptor phosphorylation in intact cells is mediated, at least in part, by CK1α.

Interestingly, the m3-muscarinic receptor is not the only GPCR found to be phosphorylated by casein kinase I in intact cells. In a recent study, stimulus-dependent phosphorylation of the α-factor pheromone receptor of Saccharomyces cerevisiae (Ste2p) was also reported to be mediated by casein kinase I, and this was associated with control of receptor ubiquitination and endocytosis (30).

The fact that agonist-mediated phosphorylation of the m3-muscarinic receptor in the present study was not completely inhibited by either F-CK1αK46R or the 3i loop peptide indicates that either the inhibitors are not expressed in sufficiently high concentrations or that phosphorylation is mediated by kinases in addition to CK1α. In the case of the GRK-2 dominant negative mutant, in vitro studies have demonstrated that a 10-fold molar excess of the dominant negative mutant was required to inhibit GRK-2-mediated β2-adrenergic receptor phosphorylation by 60% (in the presence of G-protein βγ-subunits) (16). In the present study we were only able to achieve a level of F-CK1αK46R expression that was approximately equivalent to endogenous CK1α. This level of expression may have been insufficient to inhibit completely the endogenous kinase activity.

An alternative, and attractive, proposition is that CK1α is one of a number of kinases responsible for agonist-mediated phosphorylation. There is, for example, evidence for the involvement of the GRKs in muscarinic receptor phosphorylation. The adenylyl cyclase-coupled m2-muscarinic receptor was one of the first receptors to be shown to be an in vitro substrate for the GRKs (31). Phosphorylation at sites on the third intracellular loop is thought to mediate m2-muscarinic receptor internalization (6, 32). m3-muscarinic receptors contained in urea-treated membranes have previously been shown to be phosphorylated by purified GRK-2 and -3 but not GRK-5 and -6 (11). A peptide corresponding to the third intracellular loop of the m3-muscarinic receptor has also been shown to be a substrate for GRK-2 in in vitro studies (33). Furthermore, the purified m1-muscarinic receptor is phosphorylated in an agonist-dependent manner following reconstitution with GRK-2 (12). These in vitro reconstitution studies indicate that certain GRKs have the potential to phosphorylate members of the muscarinic receptor family coupled via both G i and G αi11, G-proteins, although direct evidence that this is the case in cells has yet to be presented. Hence, placed in context with previous studies our data suggest the intriguing possibility that the m3-muscarinic receptor may be phosphorylated by CK1α and possibly one or more of the GRKs. Ongoing studies in our laboratory mapping the phosphorylation sites on the m3-muscarinic receptor may reveal phospho-acceptor sites that cannot be assigned to CK1α and thereby indicate the involvement of other receptor kinases.

The functional consequences of receptor phosphorylation were investigated in a receptor where the region Lys370–Ser425 in the third intracellular loop had been deleted. Compared with the wild type receptor this mutant showed an ~80% decrease in agonist-mediated receptor phosphorylation. The region deleted included the putative CK1α-binding site, identified in this study to reside in the domain His374–Val391. Although the reduction in the ability of the Lys370–Ser425 deletion mutant to undergo agonist-mediated receptor phosphorylation might be explained by the loss of the putative CK1α-binding site, it must be noted that this deletion also removed eight serine residues that may act as potential phospho-acceptor sites. Furthermore, the results reported here are consistent with our previous studies using bacterial fusion proteins where we showed that deletion of either Lys370–Ser425 or His374–Val391 from a bacterial fusion protein expressing the majority of the third intracellular loop (i.e. Ser345–Leu463, termed Ex-m3) resulted in a dramatic reduction in the level of phosphorylation mediated by purified CK1α (23). Functional analysis of the wild type m3-muscarinic receptor Ins(1,4,5)P 3 response revealed a characteristic peak/plateau response to agonist stimulation where the peak response at 5–10 s was desensitized by a pre-stimulation with agonist. Previously we have speculated that due to the rapid time course of agonist-mediated m3-muscarinic receptor phosphorylation, and the large weight of evidence in the literature linking receptor phosphorylation to GPCR desensitization, that phosphorylation was the mechanism underlying the desensitization of the peak Ins(1,4,5)P 3 response (20, 25, 34). To our surprise the Lys370–Ser425 deletion mutant receptor showed a peak/plateau Ins(1,4,5)P 3 response with a temporal profile very similar to the wild type receptor. Furthermore, pre-stimulation of the Lys370–Ser425 deletion mutant receptor resulted in desensitization of the peak Ins(1,4,5)P 3 response. These data suggest that agonist-mediated phosphorylation of the m3-muscarinic receptor was not involved in the desensitization of the peak Ins(1,4,5)P 3 response. It is, however, possible that the small amount of phosphorylation that remains in the Lys370–Ser425 deletion mutant receptor, possibly mediated by one or more of the GRKs (see above), may be sufficient to induce desensitization of the peak Ins(1,4,5)P 3 response.

Recent studies have identified a number of GPCRs that undergo phosphorylation-independent desensitization. For example, heterologous desensitization of the formyl-methionyl-leucyl-phenylalanine and the bradykinin B2 receptors are not associated with receptor phosphorylation (35–37). A similar lack of correlation between receptor phosphorylation and desensitization has also been reported for the chemoattractant receptor of Dictostelium where removal of the phospho-acceptor sites does not affect desensitization of the adenylyl and guanylyl cyclase responses (38). The possibility that desensitization of the m3-muscarinic receptor is a result of mechanisms downstream of the receptor is currently being pursued in our laboratory.

In the present study the Lys370–Ser425 deletion mutant receptor gave a more robust Ins(1,4,5)P 3 response when compared with the wild type receptor (2–3 fold). This was not due to changes in agonist affinities for the receptor nor changes in the efficacy of the agonist but may reflect an enhanced coupling of the receptor to G αi11. An increase in the magnitude of the Ins(1,4,5)P 3 response was also seen in cells where m3-muscarinic receptor phosphorylation was reduced by co-transfection with F-CK1αK46R, suggesting that receptor phosphorylation mediated by CK1α was able to control the magnitude of the Ins(1,4,5)P 3 response. An enhanced inositol phosphate signal has previously been reported for truncation mutants of the platelet-activating factor (39) and neurokinin-2 receptors (40).
where putative phospho-acceptor sites had been removed. In these cases receptor-stimulated phospholipase C activity appeared to be “up-regulated” by removing phospho-acceptor sites resulting in augmented signaling. It appears, therefore, that receptor phosphorylation may play a role in controlling the magnitude of the inositol phosphate response mediated by m3-muscarinic receptors and possibly other phospholipase C-coupled GPCRs.

In summary we show that inhibition of endogenously expressed CK1α reduces agonist-mediated phosphorylation of the m3-muscarinic receptor. These data support our earlier studies suggesting that CK1α is a receptor kinase involved in GPCR phosphorylation. Furthermore, by using a receptor mutant that shows reduced agonist-mediated phosphorylation, we show that phosphorylation is not associated with desensitization of the peak Ins(1,4,5)P3 response but may instead be involved in controlling the magnitude of the inositol phosphate response.

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Phosphorylation and Regulation of a $G_{q/11}$-coupled Receptor by Casein Kinase $1\alpha$

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