Activation of the Mitogen-activated Protein Kinase Pathway by a G_{q/11}-coupled Muscarinic Receptor Is Independent of Receptor Internalization*

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A number of recent studies have demonstrated an essential role for receptor endocytosis in the activation of the mitogen-activated protein (MAP) kinases, Erk-1 and Erk-2, by growth factor receptors and the G-protein coupled β2-adrenergic receptor. Because ligand-mediated receptor endocytosis and activation of the MAP kinase pathway are common phenomena among G-protein coupled receptors, it has been suggested that the essential role of endocytosis in MAP kinase activation identified for the β2-adrenergic receptor may be universal for all G-protein coupled receptors (Daaka,Y., Luttrell, L. M., Ahn, S., Della Rocca, G. J., Ferguson, S. S. G., Caron, M. G., and Lefkowitz, R. J. (1998) J. Biol. Chem. 273, 685–688). We tested this hypothesis using the G_{q/11}-coupled m3-muscarinic receptor expressed in Chinese hamster ovary cells and an m3-muscarinic receptor mutant that does not undergo endocytosis. We demonstrate that inhibition of endocytosis by concanavalin A and cytochalasin D does not affect the ability of the wild type m3-muscarinic receptor to activate Erk-1/2. Furthermore, the mutant m3-muscarinic receptor that is unable to undergo endocytosis, activates the MAP kinase pathway in an identical manner to the wild type receptor. We conclude that receptor endocytosis is not universally essential for MAP kinase activation by G-protein coupled receptors. We discuss the possibility that the differential roles played by endocytosis in MAP kinase activation between various receptor subtypes may be linked to the mechanism of upstream activation of Raf-1.

The mitogen-activated protein (MAP) kinase, Erk-1 and Erk-2, are phosphorylated and activated by a protein kinase cascade that can be initiated by both tyrosine kinase growth factor receptors and G-protein coupled receptors (GPCRs) (1, 2). Many of the same signal transduction proteins are used by both growth factor receptors and GPCRs in the activation of the MAP kinase pathway. The commonality between these two receptor types has recently been extended to include a role for ligand-mediated receptor endocytosis in the mechanism of MAP kinase activation (3, 4).

Endocytosis of growth factor receptors, via clathrin-coated pits, is known to play an essential role in Erk-1/2 activation (5, 6). Recent studies on the β2-adrenergic receptor have also demonstrated that clathrin/dynamin-mediated receptor endocytosis may also be essential in the activation of the MAP kinase pathway by GPCRs (3, 4). These studies indicated that ligand-induced β2-adrenergic receptor endocytosis provided a link between the activated Raf-1 complex and MEK, the downstream kinase in the phosphorylation cascade (3, 4). Inhibition of endocytosis was sufficient to prevent Raf-1 activation of MEK and thereby block Erk-1/2 phosphorylation mediated by the β2-adrenergic receptor (3).

Because many GPCRs undergo ligand-induced endocytosis via a clathrin/dynamin-mediated process (7) and that GPCRs activate the MAP kinase pathway via Raf-1 (2), it has been suggested that GPCRs generally employ receptor-mediated endocytosis in the mechanism of activation of the MAP kinase pathway (4). This hypothesis, however, has not been extensively tested for receptor subtypes other than the β2-adrenergic receptor. Importantly, different receptor subtypes activate the MAP kinase pathway via distinct mechanisms. These mechanisms depend on the G-protein subtype to which the GPCR is coupled (8–13). Whether the differential mechanisms by which GPCRs activate the MAP kinase pathway contribute to the role played by receptor endocytosis has not yet been examined.

To address the question of the possibility that receptor endocytosis is universally essential in the activation of the MAP kinase pathway by GPCRs, we investigate here the G_{q/11}-coupled m3-muscarinic receptor expressed in CHO cells. This receptor type shares a number of properties with the β2-adrenergic receptor in that the receptor is rapidly phosphorylated following agonist stimulation (14) and that it undergoes endocytosis in a clathrin/dynamin-dependent process (15, 16). Using inhibitors of endocytosis and a mutant receptor deficient in the ability to undergo ligand-mediated endocytosis, we investigate the role receptor endocytosis plays in the activation of Erk-1/2 by the m3-muscarinic receptor. Further, we discuss the possibility that the upstream mechanism of Raf-1 activation may play a role in determining the involvement of receptor endocytosis in GPCR-mediated Erk-1/2 activation.

**MATERIALS AND METHODS**

**Cell Culture**—Chinese hamster ovary cells (CHO) were maintained in α-minimal essential media (MEM) supplemented with fetal calf serum (10% v/v), fungizone (2.5 μg/ml), penicillin (100 IU/ml), streptomycin (100 μg/ml) at 37 °C in a humidified incubator containing 5% CO₂. CHO-m3 cells expressing recombinant human m3-muscarinic receptors (1.55 ± 0.04 pmol/mg of protein), and CHO cells expressing the m3-muscarinic receptor mutant m3A^{349AAA}AA^{352} (4.72 ± 0.08 pmol/mg protein) were kind gifts from Dr. Wolfgang Sadee and Dr. Jelveh Lameh (School of Pharmacy, University of California, San Francisco, CA). The generation of the m3A^{349AAA}AAA^{352} has been previously described (17).

In experiments where endogenous PKC was down-regulated by phor-
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bol 12,13-dibutyrate, cells were exposed to 1 μM phorbol ester overnight prior to experimentation. Furthermore, in experiments where pertussis toxin was used, cells were incubated with 100 ng/ml pertussis toxin overnight before experimentation.

Inositol 1,4,5-Trisphosphate (Ins1,4,5P3) Determination—Cells grown to confluence in 24-well plates were washed with 250 μl of Krebs-Henseleit buffer (KHB, 10 mM HEPES, pH 7.4, 118 mM NaCl, 4.3 mM KCl, 1.17 mM MgSO4·7H2O, 1.3 mM CaCl2·2H2O, 25 mM NaHCO3, 11.7 mM glucose). Cells were stimulated with 1 mM carbachol for the indicated times. Reactions were terminated by the addition of an equal volume of 1 x trichloroacetic acid, and Ins1,4,5P3 was determined by a radioreceptor assay previously described (18).

Receptor Phosphorylation—Cells grown to confluence in 6-well plates were labeled with [32P]orthophosphate (50 μCi/ml) for 1 h. Cells were stimulated with 1 mM carbachol for the times indicated, and the reactions were terminated by rapid aspiration of media and the addition of 1 ml of ice-cold RIPA buffer (10 mM Tris, 500 mM NaCl, 10 mM EDTA, 1% v/v Nonidet P-40, 0.1% v/v SDS, 0.5% w/v Na-deoxycholate). The mutant m3(A349AAA352) receptor expression levels were 3-fold greater than wild type receptors. Therefore, to ensure that equivalent numbers of receptors were immunoprecipitated, the proteins were adjusted so that ~1 pmol of solubilized receptors were used for each immunoprecipitation. The receptors were immunoprecipitated from precleared lysates using 1 μg of anti-muscarinic m3 receptor antibody (14). Immunoprecipitates were collected on protein-A-Sepharose beads and the beads were washed three times with ice-cold TE buffer (10 mM Tris, pH 7.4, 2.5 mM EDTA). Immunocomplexes were resuspended in 2x SDS-polyacrylamide gel electrophoresis sample buffer and placed in a boiling water bath for 2 min. Proteins were resolved on an 8% SDS-polyacrylamide electrophoresis gel. The gels were stained with 0.2% Coomassie Blue to ensure that there was equal immunoprecipitation. The gels were then dried and phosphorylated bands were visualized by autoradiography.

Receptor Density Determination—Confluent CHO cells grown in 24-well plates were serum-starved in KHB and then stimulated with the appropriate agents. Stimulations were terminated by aspiration, and the cells were washed three times with ice-cold KHB. Cells were incubated with ~0.14 μCi of [3H]N-methylscopolamine (NMS) overnight at 4°C. Cells were washed two times in ice-cold KHB and solubilized by the addition of 1 ml of ice-cold RIPA buffer, and receptor number was determined by liquid scintillation counting. Nonspecific binding was determined by the inclusion of 10 μM atropine.

Erk-1/2 Assay—CHO cells grown to confluence in 6-well plates were serum-starved in KHB and then stimulated with the appropriate agents. Stimulations were terminated by aspiration, and cells were incubated for 10 min in lysis buffer (20 mM Tris, pH 7.6, 0.5% Nonidet P-40, 250 mM NaCl, 3 mM EDTA, 3 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na3VO4, 1 mM diithiothreitol, 5 μM/ml benzamidine) at 4°C. Solubilized CHO cell lysates were pre-cleared by centrifuging at 20,000 × g for 5 min. Endogenous MAP kinase was immunoprecipitated using 5 μl of anti-Erk-1/2 antiserum (Santa Cruz Biotechnology). Protein A-Sepharose-immobilized MAP kinase was washed twice in lysis buffer and twice in assay buffer (20 mM HEPES, pH 7.2, 20 mM β-glycerophosphate, pH 7.2, 10 mM MgCl2, 1 mM diithiothreitol, 50 μM Na3VO4). Washed pellets were resuspended in assay buffer containing 2 μCi [32P]ATP, 20 μM ATP, 200 μM EGFr (peptide encompassing region 661-681 of EGFr 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. 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.

Assay of Raf-1 Kinase Activity—Confluent CHO cells seeded in 6-well plates were serum-starved for 1 h in KHB and stimulated with 1 mM carbachol for the appropriate times. Reactions were terminated by rapidly aspirating the medium, and the cells were lysed in cold lysis buffer (GLB, composition: 20 mM Tris, pH 8.0, 137 mM NaCl, 5 mM EDTA, 1 mM EGTA, 15% glycerol, 1% (v/v) Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 50 μg/ml benzamidine, 1 mM Na3VO4, 10 mM β-glycerophosphate, 1 mM Na4P2O7). Setting the [32P]ATP (kind gift from Dr. C. Fridehard, Dept. of Biochemistry, University of Leicester, UK). Immunocomplexes were washed two times with ice-cold GLB and two times with ice-cold Raf-1 assay buffer (25 mM HEPES, pH 7.2, 10 mM MgCl2, 1 mM diithiothreitol, 1 mM MnCl2). Washed immunocomplexes were resuspended in Raf-1 assay buffer containing 20 μM ATP, 2 μCi [32P]ATP, 1.8 μg of Raf-1 peptide substrate. Assays were subsequently performed as for the Erk assay.

RESULTS

Effect of Inhibition of Receptor Endocytosis on m3-Muscarinic Receptor Erk-1/2 Activation—Stimulation of CHO-m3 cells expressing the recombinant human m3-muscarinic receptor with the cholinergic agonist carbachol (60 min, 1 mM) resulted in a 56% loss of cell surface receptors as determined using the binding of the hydrophilic ligand [3H]NMS (Fig. 1). Total receptor number determined using [3H]NMS binding to a broken cell preparation from stimulated or control cells remained unchanged during this time (data not shown), indicating that the loss of receptor binding at the cell surface was because of receptor endocytosis (or internalization) rather than receptor degradation. m3-Muscarinic receptor endocytosis could be completely inhibited following pretreatment with concanavalin A and reduced by 81% by cytochalasin D (Fig. 1).

Stimulation of m3-muscarinic receptors resulted in an ~25-fold increase in Erk-1/2 activity within 5 min, which fell to ~8.5-fold within 20 min (Fig. 2). Inhibition of m3-muscarinic receptor endocytosis using concanavalin A and cytochalasin D had no significant effect on m3-muscarinic receptor activation of Erk-1/2 (Fig. 2).

Characterization of the m3(A349AAA352) Receptor Mutant—Previous studies have demonstrated that mutation of a region in the third intracellular loop of the m3-muscarinic receptor (S349A350A352) was associated with the cholinergic agonist carbachol (60 min, 1 mM) resulted in a receptor that was unable to undergo receptor endocytosis (17). This was confirmed in the present study where expression of the m3(A349AAA352) mutant in CHO cells resulted in a receptor that showed no significant endocytosis following 60 min of agonist treatment (Fig. 3A). In contrast, wild type receptors were internalized by 51.1 ± 5.1% following 60-min stimulation with carbachol (Fig. 3A). The m3(A349AAA352) receptor did, however, couple to the phospholipase C (PLC) pathway as evident by a peak and plateau profile of Ins(1,4,5)P3 production (Fig. 3B). Comparison of the peak Ins(1,4,5)P3 production revealed a numerical but not significant reduction in insulin (1,4,5)P3 for the m3(A349AAA352) receptor compared with wild type receptor (p = 0.078, n = 6, unpaired Student’s t test). Furthermore, the m3(A349AAA352) receptor also underwent rapid agonist-mediated phosphorylation (Fig. 3C), which was maximal after ~30 s and was maintained for at least 30 min (data not shown). Densitometric
analysis revealed that phosphorylation of the m3(A349AAA352) receptor was ∼3-fold greater than the wild type receptor. Note that in the data presented in Fig. 3C, the receptor number was normalized at the point of immunoprecipitation so that an equal number of wild type and m3(A349AAA352) mutant receptors were immunoprecipitated (see "Materials and Methods").

Activation of Erk-1/2 Activity by the Wild Type and m3(A349AAA352) Receptor—Despite the fact that the m3(A349AAA352) receptor did not undergo endocytosis, the m3(A349AAA352) receptor stimulated Erk-1/2 activation with a time course that was similar to the wild type receptor (Fig. 4A). The m3(A349AAA352) receptor did, however, show a slight lag evident at 1 min (p < 0.05) and a significantly more robust response at 20 min (p < 0.01) when compared with the wild type receptor. The agonist-concentration dependence between the wild type and m3(A349AAA352) receptor was not significantly different (p = 0.82, two-way analysis of variance) (Fig. 4B). The wild type and m3(A349AAA352) receptor activation of Erk-1/2 could be completely blocked by the MEK-1/2 inhibitor PD98053 (Calbiochem, data not shown). Furthermore, both the wild type and m3(A349AAA352) receptor Erk-1/2 responses were mediated, at least in part, by PKC. Down-regulation of PKC using phorbol ester pretreatment reduced the wild type and m3(A349AAA352) receptor responses by 87 and 77%, respectively (Fig. 5). In control experiments, Ro-318220 completely inhibited the Erk-1/2 response to phorbol 12,13-dibutyrate (data not shown).

Previous studies have demonstrated that Gq/11-coupled muscarinic receptors expressed in CHO cells activated Erk-1/2 via a pertussis toxin-insensitive G-protein (11, 13). This was confirmed in the present study where both m3(A349AAA352) and wild type receptor-stimulated Erk-1/2 activity was unaffected by pertussis toxin pretreatment (Fig. 5).

The ability of the muscarinic receptor to activate Raf-1, the upstream kinase in the MAP kinase cascade, was also investigated. m3-Muscarinic receptor activation stimulated an ∼5-
fold increase in Raf-1 kinase activity (basal = 125 fmol of phosphate incorporated/mg/min, n = 2) that peaked at 1–5 min followed by a plateau phase (~3-fold over basal) evident at 10 min and extending for at least 20 min.

**DISCUSSION**

The present study demonstrates that the G_{q11}-coupled m3-muscarinic receptor expressed in CHO cells activates the MAP-kinase pathway (Erk-1/2) via a mechanism that is independent of receptor endocytosis. These data conflict with recent reports, among GPCRs (7) and that GPCR activation of the MAP-kinase pathway occurs through Raf-1 (2), it has been suggested that the role for receptor endocytosis in Erk-1/2 activation described for the β2-adrenergic receptor may generally be applicable to all GPCRs (4). Despite this, however, the data presented here clearly demonstrate that activation of the MAP-kinase pathway by m3-muscarinic receptors does not require receptor endocytosis.

Muscarinic receptors coupled via G_{q11} to the PLC pathway have previously been shown to undergo endocytosis via a clathrin/dynamin pathway (15, 16, 23). Consistent with these findings are data presented here that demonstrate the ability of cancanavalin A and cytochalasin D, reagents known to disrupt clathrin-mediated endocytosis (24), to inhibit m3-muscarinic receptor internalization. Furthermore, we have previously reported that the m3-muscarinic receptor is rapidly phosphorylated following agonist stimulation (14), possibly by CK1α (25). The role phosphorylation plays in m3-muscarinic receptor endocytosis is unclear. It is interesting to note, however, that in the present study we show that the process of receptor phosphorylation is not sufficient to ensure endocytosis since, despite the m3(A^{349}AA^{352}) receptor mutant undergoing agonist-mediated phosphorylation, this receptor is unable to endocytose. In fact, the m3(A^{349}AA^{352}) receptor was phosphorylated to a level ~3-fold greater than the wild type receptor. The apparent hyper-phosphorylation of the m3(A^{349}AA^{352}) receptor may be explained by findings from previous studies that suggest a role for receptor endocytosis in dephosphorylation of GPCRs (26). If endocytosis is important for muscarinic receptor dephosphorylation, then a mutant receptor unable to undergo endocytosis may “accumulate” in the phosphorylated form. Whether this is the reason for the hyper-phosphorylation of the m3(A^{349}AA^{352}) receptor is presently under investigation.

The m3-muscarinic receptor expressed in CHO-m3 cells, therefore, shares some characteristics in common with the β2-adrenergic receptor that is known to be important in β2-adrenergic receptor-mediated MAP-kinase activation. Namely, the receptor undergoes rapid phosphorylation following agonist stimulation and is internalized via a clathrin/dynamin-dependent pathway. However, despite these similarities, the receptors
FIG. 5. Characterization of Erk-1/2 activation by wild type m3-muscarinic and m3(A249-AAA252) receptors. Panels A–C represent wild type m3-muscarinic receptors, and panels D–F represent m3(A249-AAA252) receptors. A and D, cells were either treated overnight (~12 h) with phorbol 12,13-dibutyrate (1 μM, PDBu) or with vehicle. Cells were then stimulated with carbachol (1 mM, CCH), phorbol 12,13-dibutyrate (1 μM, PDBu), or vehicle for 5 min. Cells were then lysed and Erk-1/2 activity determined. B and E, cells plated on 6-well dishes were stimulated for 5 min with carbachol (1 mM, CCH) or vehicle. In the appropriate experiments, the PKC inhibitor Ro-31-8220 (10 μM, Ro) was added 10 min before the stimulatory agents. The reaction was then stopped by addition of lysis buffer. Erk-1/2 was immunoprecipitated from the lysate and used in an in vitro kinase assay to determine the activity of Erk-1/2. C–F, cells were either treated overnight with pertussis toxin (100 ng/ml, FTX) or vehicle. Cells were stimulated for 5 min with carbachol (1 mM, CCH) and then lysed, and Erk-1/2 activity was determined. Basal levels of Erk-1/2 activity in control cells was 145.5 ± 23.5 fmol/mg/min and for pertussis toxin-treated cells 101.6 ± 4.1 fmol/mg/min. The data presented represent the mean ± S.E. for three experiments carried out in duplicate.

do not share a common role for receptor endocytosis in MAP-kinase activation.

The m3-muscarinic and β2-adrenergic receptors do show divergent mechanisms of activation of the MAP-kinase pathway at the level of the upstream activation of Raf-1. In some cell types, Gq/11-coupled receptors including muscarinic receptors, have been shown to activate Erk-1/2 in a pertussis toxin-insensitive manner, via PKC, in a process that is independent of Ras but dependent on Raf-1 (11, 12, 13). The data presented here demonstrates that the m3-muscarinic receptor-stimulated Erk-1/2 activity is reduced by PKC inhibition and is unaffected by pertussis toxin, suggesting that a similar mechanism is in operation for the m3-muscarinic receptor expressed in CHO-m3 cells. Furthermore, the ability of the m3-muscarinic receptors to activate Raf-1 in this study indicates that the process of Erk-1/2 activation by m3-muscarinic receptors in CHO-m3 cells is mediated via Raf-1 activation of MEK. This is supported by the fact that the MEK inhibitor PD98059 blocked m3-muscarinic receptor-mediated Erk-1/2 activation. In contrast with the Gq/11-coupled muscarinic receptor subtypes, the mechanism of Raf-1 activation by β2-adrenergic receptors involves β2-subunits derived from pertussis toxin-sensitive G-proteins activating tyrosine kinases that are able to phosphorylate adapter proteins involved in the recruitment of Ras exchange factors and the activation of Ras (21). The common thread, therefore, in the activation of Erk-1/2 by GPCR subtypes, and indeed growth factor receptors, is that they all operate through the activation of Raf-1. The argument tested in this report is as follows. If receptor endocytosis is essential for Raf-1 activation of MEK, which appears to be the case for some growth factor receptors (5, 6) and the β2-adrenergic receptor (3), then it might be predicted that this would be true for GPCRs generally because they undergo ligand-dependent endocytosis and all stimulate Erk-1/2 via Raf-1. Our data, however, do not support this hypothesis and demonstrate that receptor endocytosis is not universally essential in the mechanism of GPCR activation of the MAP kinase pathway. The reason for the differences in the role played by endocytosis between the various GPCR subtypes may lie at the differential upstream mechanisms employed in the activation of Raf-1, e.g. whether via PKC or β2-subunits.

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