The m3 muscarinic acetylcholine receptor is coupled to mitogen-activated protein kinase via protein kinase C and epidermal growth factor receptor kinase

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The acetylcholine analogue carbachol rapidly activated mitogen-activated protein kinase (MAPK), and caused tyrosine phosphorylation of the adapter protein p52 Shc and the epidermal growth factor (EGF) receptor, in human embryonic kidney cells stably expressing m3 muscarinic receptors. The protein kinase C (PKC) inhibitor GF109203X caused a significant partial inhibition of m3 receptor-mediated activation of MAPK. The PKC-independent MAPK activity elicited by carbachol in the presence of GF109203X was reproducibly abolished by AG1478, an inhibitor of EGF-receptor tyrosine kinase activity, and by the Src tyrosine kinase inhibitor PP1. In a subset of these experiments, GF109203X concomitantly increased carbachol-induced tyrosine phosphorylation of p52 Shc and the EGF receptor. In co-

stimulation experiments, carbachol and EGF activated MAPK in a non-additive fashion; moreover, EGF-induced association of Shc with the phosphorylated EGF receptor was inhibited by carbachol. This effect of carbachol was blocked by GF109203X. The results indicate that MAPK activation by m3 receptor stimulation is regulated by two pathways; one dependent on PKC, and the other mediated via the EGF receptor and Src. Moreover, the EGF-receptor-dependent pathway may be subject to negative-feedback regulation via m3 receptor-coupled activation of PKC.

Key words: bisindolylmaleimide, carbachol, Shc, Src, trans-activation.

INTRODUCTION

Activation of the mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) cascade is an important consequence of cell stimulation by growth factors, by ligands for receptors coupled to G-proteins and by extracellular matrix proteins that are ligands for integrins [1–3]. The MAPK family members p42 MAPK (ERK 2) and p44 MAPK (ERK 1) are key regulators of cell proliferation, migration, differentiation, transformation and survival [4–8]. The canonical MAPK-activation pathway initiated by growth factors involves recruitment of the adapter protein Grb2 in association with the Ras guanine nucleotide-exchange protein mSos to the activated, auto-phosphorylated growth factor receptor, with subsequent sequential activation of Ras, Raf, MAPK kinase (MEK 1/2) and MAPK [9].

It was recognized subsequently that G-protein-coupled receptors (GPCRs), including members of the muscarinic acetylcholine receptor family, are functionally linked to the MAPK cascade via several distinct pathways. Of the five muscarinic receptor subtypes that have been cloned, only m1, m3 and m5 receptors are coupled efficiently to phosphatidylinositol 4,5-bisphosphate hydrolysis [10,11]. Muscarinic m1 receptors have been reported to activate MAPK via a pathway that involves pertussis toxin-insensitive $G_{q/11}$, or pertussis toxin-sensitive G_o G-proteins, protein kinase C (PKC) and Raf, but which is independent of Ras [12,13]. Others describe a pathway that, while dependent on Raf, and independent of Ras, is only partially PKC-dependent [14]. Some reports have implicated $\beta\gamma$ subunits

in Ras-dependent activation of MAPKs by m1 receptors [15,16], whereas other investigators have found that the response to m1 receptor stimulation is not affected by inhibitors of $\beta\gamma$ signalling [12]. Activation of MAPKs by m2 muscarinic receptors is also reportedly dependent on release of $\beta\gamma$ subunits from pertussis toxin-sensitive G-proteins, with subsequent tyrosine phosphorylation of Shc, which in turn associates with Grb2–mSos, leading to Ras activation [17–19]. The link between $G_{\beta\gamma}$ release and Shc phosphorylation may involve sequential activation of phosphatidylinositol 3-kinase γ and a Src-like tyrosine kinase [19–21].

Typically, activation of MAPK by GPCRs involves tyrosine phosphorylation of one or more proteins that act as scaffolds, forming complexes with Grb2–mSos, and thereby promoting the activation of the Ras-MAPK cascade [22]. One such scaffolding protein is the epidermal growth factor (EGF) receptor. EGF-independent activation of this receptor by GPCRs, a phenomenon referred to as ‘transactivation’ [21,23–28], results in the formation of a complex composed of the phosphorylated EGF receptor and the adapter proteins Shc and Grb2 [23,26,27]. The adapter proteins mediate the recruitment of mSos to the complex, thus recapitulating the sequence of events set into motion by direct activation of growth factors by their ligands. Other evidence implicates the focal adhesion-associated protein focal adhesion kinase (FAK) [22], and another member of this family, PYK2 [29–33], as additional scaffolds for the assembly of a Ras activation complex, and activation of MAPK, in response to GPCR ligands. More information about these signalling mechanisms may be found in two recent reviews [34,35].

Abbreviations used: DMEM, Dulbecco’s modified Eagle’s medium; EGF, epidermal growth factor; ERK, extracellular signal-related kinase; GPCR, G-protein-coupled receptor; HEK, human embryonic kidney; MAPK, mitogen-activated protein kinase; PKC, protein kinase C; PP1, 4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo-*d*-3,4-pyrimidine.

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In the present study the regulation of MAPK by muscarinic m3 receptors was examined in human embryonic kidney (HEK) cells stably expressing m3 receptors. The results indicate that the m3 receptor is coupled to MAPK via both PKC-dependent and EGF receptor-dependent pathways.

EXPERIMENTAL

Materials

Antibodies and other reagents were obtained from the following sources. Anti-paxillin, anti-phosphotyrosine (clone PY20), monoclonal anti-Shc and goat peroxidase-linked anti-mouse IgG were from Transduction Laboratories (Lexington, KY, U.S.A.). Monoclonal peroxidase-linked anti-phosphotyrosine clone PY99 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Polyclonal anti-Shc and anti-EGF-receptor antibodies were purchased from Upstate Biotechnology (Lake Placid, NY, U.S.A.). A polyclonal antibody specific for the activated form of MAPK was obtained from New England Biolabs (Beverly, MA, U.S.A.). Protein G agarose was purchased from Oncogene Science (Cambridge, MA, U.S.A.). Protein A-Sepharose was obtained from Pharmacia (Piscataway, NJ, U.S.A.). Reagents, equipment and mini-gels for electrophoresis were supplied by Bio-Rad Laboratories (Hercules, CA, U.S.A.). Carbamylcholine chloride (carbachol), PMA and mouse EGF were obtained from Sigma (St. Louis, MO, U.S.A.). The tyrphostin AG1478, bisindolylmaleimide I (GF109203X), bisindolylmaleimide IX (Ro 31-8220) and the Src inhibitor PPI [4-amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo-*b*-3,4-pyrimidine] were purchased from Alexis Biochemicals (San Diego, CA, U.S.A.). The indolocarbazole Gö6976 was obtained from Calbiochem (San Diego, CA, U.S.A.). Other reagents were obtained from Sigma or from Fisher Scientific (Pittsburgh, PA, U.S.A.).

Cell culture

HEK cells stably transfected with muscarinic m3 receptors were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % fetal bovine serum [36]. These cells were shown previously to express approx. 200 000 m3 receptors per cell [11]. Cells were grown on 60- or 100-mm culture dishes (Falcon; Becton Dickinson Labware, Bedford, MA, U.S.A.) coated with poly-D-lysine for 3–4 days prior to an experiment. Cultures were maintained in growth medium until the day of the experiment. All pharmacological treatments were carried out in serum-free DMEM. Stock solutions of inhibitors and PMA were dissolved in DMSO, and diluted with culture medium prior to treatment of cells.

Immunoprecipitation and determination of tyrosine-phosphorylated proteins

Following incubation, test media were aspirated and cells were collected in 1 ml of lysis buffer containing 1 % Nonidet P-40, 0.1 % SDS, 0.5 % deoxycholate, 50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM sodium orthovanadate, 25 mM NaF, 2 mM 4-(2-aminoethyl)benzenesulphonyl fluoride (AEBSF), 1 µg/ml leupeptin and 2 µg/ml aprotinin. Lysates were centrifuged to remove insoluble material, normalized for protein content, and incubated overnight with immunoprecipitating antibodies, usually at concentrations of 2–4 µg/500 µg of protein, and with Protein A-Sepharose or Protein G agarose (3.5 mg per sample). Immunoprecipitates were centrifuged and washed three times in a washing buffer containing 0.1 % Triton X-100, 25 mM Tris, pH 7.5,

250 mM NaCl and 1 mM sodium orthovanadate. Immunoprecipitates were size-fractionated by SDS/PAGE, and the proteins were transferred on to PVDF membranes. The membranes were blocked with 3 % gelatin or 5 % milk in Tris-buffered saline containing 0.15 % Tween-20. Tyrosine-phosphorylated proteins were immunoprecipitated with anti-phosphotyrosine antibodies (clone PY20). Immunoblots were probed with recombinant peroxidase-linked anti-phosphotyrosine (PY99) antibodies, or antibodies to paxillin or Shc. Activated MAPK was detected in cell lysates by immunoblotting with a phosphorylation-specific antibody (New England Biolabs). Approximate molecular masses were estimated using calibrated prestained standards (Bio-Rad Laboratories).

Statistical analysis

Comparisons based on immunoblotting data were derived from samples processed on the same blot. The statistical significance of differences was estimated by analysis of variance followed by Fisher's least-significant-difference (LSD) test. Differences were taken to be significant at $P < 0.05$.

RESULTS

MAPK is activated by carbachol and EGF in HEK cells expressing m3 receptors

In HEK cells expressing muscarinic m3 receptors, carbachol caused a concentration-dependent increase in MAPK activity. The response was detected using an antibody specific for the activated, tyrosine- and threonine-phosphorylated form of the enzyme (Figure 1A). The activation was apparent within 2 min, and reached a maximum value by 5 min (Figure 1B). EGF at a concentration of 50 ng/ml, acting on endogenous EGF receptors, stimulated MAPK as effectively as carbachol (Figure 1A).

PKC inhibitors reduce m3 receptor-mediated MAPK activation

A number of different mechanisms regulate MAPK activation by GPCR ligands. The response in some cell types is dependent on

Figure 1 Stimulation of m3 muscarinic receptors increases MAPK activity

(A) HEK cells stably expressing the m3 muscarinic receptor were treated for 10 min with various concentrations of carbachol (carb), or with 300 ng/ml EGF. (B) Cells were treated with 100 µM carbachol for various periods of time. Cell lysates were immunoblotted with polyclonal antibodies specific for phosphorylated, activated MAPK.

Figure 2 PKC inhibition reduces m3 receptor-coupled MAPK activation

(A) Cells were pretreated for 30 min with serum-free medium containing GF109203X (2.5 μ M) or the vehicle DMSO. Cells were then incubated for 10 min in fresh medium containing GF109203X or DMSO with or without carbachol (100 μ M) or EGF (50 ng/ml). Phosphorylated MAPK was detected in cell lysates by immunoblot analysis. (B) Cells were pretreated with DMSO or GF109203X for 30 min, then incubated for 10 min in fresh medium containing DMSO or the inhibitor with or without PMA (1 μ M).

PKC [12,13]. When the PKC inhibitor GF109203X (bisindolylmaleimide I) was tested in our model, a partial reduction in carbachol-induced MAPK activation was observed. Although the degree of inhibition observed was variable (ranging from 9 to 96%), an average reduction of $65 \pm 5\%$ (mean $\pm$ S.E.M., $n = 21$) was induced by a concentration of GF109203X (2.5 μ M) that abolished the response to the PMA (Figures 2 and 6A, see below). In contrast, EGF-induced MAPK activation was consistently resistant to this inhibitor (Figures 2A and 6B). Two additional PKC inhibitors were also tested; the indolocarbazole Gö6976 and the bisindolylmaleimide Ro31-8220. Gö6976, a selective inhibitor of the calcium-dependent PKC isozymes α and β 1 [37], inhibited PMA- but not carbachol-induced MAPK activation (Figure 3A). Ro31-8220, which inhibits both calcium-dependent and calcium-independent PKC subtypes [38], as well as the atypical subtype PKC ζ [39], markedly inhibited carbachol-induced MAPK activation at a concentration (0.3 μ M) that abolished the response to PMA (Figure 3B).

PKC-independent MAPK activation by carbachol depends on transactivation of the EGF receptor

It has been reported that stimulation of certain GPCRs transactivates the EGF receptor, leading to stimulation of MAPK, apparently via recruitment of Shc, which couples the activated receptor to mSos [23–28,33,40]. The possibility that this mechanism might account for the PKC-independent stimulation of MAPK by carbachol was addressed using a specific inhibitor of EGF receptor tyrosine kinase activity, AG1478 [41]. This inhibitor abolished MAPK activation by EGF, as expected (Figure 4A), but did not significantly inhibit carbachol-mediated tyrosine phosphorylation of paxillin, a substrate of Src [42,43] (results not shown), supporting the selectivity of AG1478 for the EGF

Figure 3 Effects of Ro31-8220 and Gö6976 on stimulation of MAPK by carbachol

(A) Cells were pretreated with DMSO or Gö6976 (5 μ M) for 30 min, then treated for 10 min with fresh medium containing DMSO or the inhibitor, alone or with carbachol (100 μ M), or PMA (1 μ M). (B) Cells were pretreated with DMSO or various concentrations of Ro31-8220 for 30 min, then treated for 10 min with fresh medium containing carbachol (100 μ M) or PMA (1 μ M) in the presence of DMSO and/or the inhibitor. Final DMSO concentrations were the same in all test solutions. The control received DMSO alone. Phosphorylated MAPK was detected in cell lysates by immunoblot analysis.

receptor tyrosine kinase. AG1478 alone caused a consistent partial inhibition (of approx. 35%) of MAPK activation by carbachol. Notably, GF109203X and AG1478 in combination virtually abolished the activation of MAPK by muscarinic receptor stimulation (Figure 4B). The results suggest that the MAPK activity remaining in the presence of carbachol and GF109203X might be due to the activity of a second pathway dependent upon EGF receptor activation.

The adapter protein Shc has been implicated in the activation of MAPK following EGF receptor activation by its cognate ligand EGF or by GPCRs [34,44]. Although three isoforms of Shc have been described, only two of these, p46 and p52 Shc, were detectable in HEK cells expressing m3 receptors. The p46 isoform was tyrosine phosphorylated under basal conditions, and did not exhibit any consistent change in phosphorylation with exposure to carbachol. In contrast, tyrosine phosphorylation of p52 Shc was increased 3.6 ± 0.7 -fold by carbachol (mean $\pm$ S.E.M., $n = 10$; Figure 4C). An increase in tyrosine phosphorylation of the EGF receptor was also observed (Figure 4C). In a subset of these experiments, carbachol-induced tyrosine phosphorylation of p52 Shc and the EGF receptor was increased further in the presence of GF109203X (Figure 4D). These effects were abolished by AG1478 (Figure 4D). In other experiments, GF109203X instead caused a partial inhibition of Shc and EGF receptor tyrosine phosphorylation. The reasons for this inconsistency have not been determined; however, it was noted that GF109203X tended to increase carbachol-mediated Shc tyrosine phosphorylation in those experiments in which it was comparatively less effective as an inhibitor of MAPK activation.

Src is a mediator of PKC-independent MAPK activation by carbachol

Src has been implicated in the activation of MAPK by certain GPCRs [22,24]. In the present study, the Src inhibitor PP1 reduced carbachol-evoked MAPK activation by approx. 50%

Figure 4 Activation of MAPK by m3-receptor stimulation is dependent on PKC and EGF-receptor transactivation

(A) Cells were pretreated for 30 min in serum-free medium containing AG1478 (250 nM) or DMSO, then treated for 10 min in fresh medium containing DMSO or the inhibitor, with or without EGF (50 ng/ml). (B) Cells were pretreated for 30 min with serum-free medium containing GF109203X (GF109; 2.5 μ M) or AG1478 (250 nM) alone or in combination, or the vehicle DMSO. Cells were then incubated for 10 min in fresh medium containing one or both inhibitors, or DMSO, with or without carbachol (100 μ M). Phosphorylated MAPK was detected by immunoblot analysis (upper panel) and quantified by densitometry (lower panel). Results represent means $\pm$ S.E.M. from 3–9 experiments. *, Significantly different from the corresponding control group; **, significantly different from carbachol alone, $P < 0.05$. (C) Cells were treated with carbachol (carb; 100 μ M) or control (con) medium for 10 min. Immunoprecipitates (IP) were prepared using antibodies to Shc (left panel), or the EGF receptor (EGFR; right panel), and analysed by immunoblotting with a peroxidase-linked anti-phosphotyrosine antibody (clone PY99). (D) Cells were treated as in (B). Immunoprecipitates were prepared using polyclonal antibodies to the EGF receptor (upper panel), or to Shc (middle and lower panels) and immunoblotted using anti-phosphotyrosine antibodies (clone PY99) (upper and middle panels) or monoclonal antibodies to Shc (lower panel). In the experiment shown, GF109203X reduced MAPK activation by 31%.

(Figure 5A). In the same series of experiments, GF109203X reduced the mean response to carbachol by 76%. The combined action of PP1 and GF109203X abolished MAPK stimulation by carbachol (Figure 5A). PP1 reduced the tyrosine phosphorylation of Shc elicited by EGF, and decreased the association of the tyrosine-phosphorylated EGF receptor with Shc in EGF-treated cells (Figure 5B, middle panel). PP1 also blocked the tyrosine phosphorylation of p52 Shc induced by carbachol (Figure 5C). The concentration of PP1 used in these experiments (10 μ M) did not affect tyrosine phosphorylation of the EGF receptor induced by EGF (Figure 5B, upper panel), but effectively inhibited carbachol-mediated tyrosine phosphorylation of paxillin, a Src substrate (results not shown). Similar results were described by Daub and colleagues [24].

Role of PKC in suppression of EGF-mediated signalling by carbachol

The results of the experiments described in this report suggest that EGF and m3 receptors might activate MAPK by at least partially convergent pathways. Therefore an experiment was carried out to determine if carbachol and EGF exert additive effects on MAPK activity. Interestingly, the responses to the two agonists were completely non-additive (Figure 6A). The PKC inhibitor GF109203X reduced the activation of MAPK by

carbachol, as shown earlier, but did not inhibit the response to EGF alone, or to EGF in combination with carbachol. The non-additive nature of MAPK stimulation by carbachol and EGF may be due to an inhibitory effect of carbachol on EGF signalling. Specifically, in the presence of carbachol, EGF-induced association of the tyrosine-phosphorylated EGF receptor with Shc was inhibited. The effect was reversed by GF109203X, suggesting that carbachol inhibits EGF signalling by activating PKC (Figure 6B).

DISCUSSION

The results described in this study demonstrate that the regulation of MAPK activity by muscarinic receptors is dependent on PKC- and EGF receptor-mediated signalling pathways. A number of studies have shown that PKC plays a role in the coupling of GPCRs, including muscarinic m1 and m3 receptors, to MAPK. PKC inhibitors, or down-regulation of PKC by long-term exposure to phorbol esters, completely inhibited MAPK activation in response to carbachol in Chinese hamster ovary cells transfected with m1 receptors [12,13], and in SH-SY5Y and SK-N-BE2(C) human neuroblastoma cells, which express endogenous m3 receptors [45,46]. However, the stimulation of MAPK by carbachol in NIH 3T3 cells transfected with m1 receptors was

Figure 5 Src acts downstream of the EGF receptor to mediate MAPK activation by carbachol

(A) Cells were pretreated for 30 min with serum-free medium containing GF109203X (2.5 μ M) or PP1 (10 μ M), alone or in combination, or the vehicle DMSO. Cells were then incubated for 10 min in fresh medium containing one or both inhibitors, or DMSO, with or without carbachol (100 μ M). Phosphorylated MAPK was detected by immunoblot analysis (upper panel) and quantified by densitometry (lower panel). Results represent means $\pm$ S.E.M. from 3 experiments. *, Significantly different from the corresponding control group; **, significantly different from carbachol alone, $P < 0.05$. (B) Cells were pretreated for 30 min with medium containing DMSO or PP1 (10 μ M) then incubated for 10 min in fresh medium containing DMSO or PP1, with or without EGF (50 ng/ml). Immunoprecipitates (IP) of Shc proteins or the EGF receptor (EGFR) were prepared, and then immunoblotted with the anti-phosphotyrosine antibody PY99. (C) Cells were pretreated for 30 min with medium containing DMSO or PP1 (10 μ M) then incubated for 10 min in fresh medium containing DMSO or PP1, with or without carbachol (100 μ M). Immunoprecipitates of Shc proteins were prepared, and immunoblotted with the anti-phosphotyrosine antibody PY99.

Figure 6 Carbachol and EGF exert non-additive effects on MAPK activity

(A) Cells were pretreated for 30 min with serum-free medium containing GF109203X (2.5 μ M) or the vehicle DMSO. Cells were then incubated for 10 min in fresh medium containing GF109203X or DMSO with or without carbachol (100 μ M) and/or EGF (50 ng/ml). Phosphorylated MAPK was detected in cell lysates by immunoblot analysis (upper panel). Bands of phosphorylated MAPK were quantified by densitometry (lower panel; carb, carbachol). Results are expressed as means $\pm$ S.E.M. from 3–8 experiments. *, Significantly different from carbachol alone, $P < 0.05$. (B) Cells were treated as in (A). Anti-Shc immunoprecipitates (IP) were prepared, and analysed by immunoblotting with anti-phosphotyrosine (upper panel) or anti-Shc (lower panel). The numbers to the right of the blot represent molecular masses in kDa.

only partially inhibited by GF109203X at a concentration (1 μ M) that nearly abolished the response to the PMA [14], indicating that an alternate pathway may mediate the response in some cell types. In the present study, which employed as a model HEK cells stably transfected with m3 muscarinic receptors, the PKC inhibitor GF109203X caused a partial reduction in carbachol-induced MAPK activation. Another PKC inhibitor, the bis-indolylmaleimide Ro31-8220, also substantially reduced MAPK activation by carbachol at a concentration that abolished the response to PMA. In contrast, the indolocarbazole Gö6976 did not affect the stimulation of MAPK by carbachol, although it too reduced the response to PMA. GF109203X and Ro31-8220 are inhibitors of both calcium-dependent and calcium-independent PKC isoforms, whereas Gö6976 inhibits only calcium-dependent isoforms [37,38]. Taken together, the results suggest that calcium-dependent and -independent PKC isoforms are both capable of regulating MAPK activity in HEK cells. That Gö6976 was ineffective against carbachol implies a role for calcium-independent PKCs as mediators of MAPK activation by m3 receptors, but does not rule out the possibility that calcium-dependent isoforms are also involved, since inhibition of their effects by Gö6976 could be masked by the activity of isoforms resistant to this inhibitor. A recent study in SK-N-BE2(C) human neuroblastoma cells showed that, although these cells express multiple PKC isoforms, only PKC ϵ was activated by carbachol, and was determined to mediate MAPK activation by carbachol [46]. This is surprising, since the stimulation of phosphoinositide hydrolysis by muscarinic receptor stimulation would be expected to activate calcium-dependent as well as calcium-independent PKC isoforms [47], and indeed, stimulation of m3 receptors in transfected Chinese hamster ovary cells activated PKC isoforms of both classes [48]. Additional experiments will be necessary to determine which specific PKC isoforms mediate the activation of MAPK by m3 receptor stimulation in the present model.

The PKC-independent MAPK activity elicited by carbachol in the presence of GF109203X was abolished either by an inhibitor of EGF receptor tyrosine kinase activity, or by an inhibitor of Src, suggesting the involvement of these two tyrosine kinases in this component of the response. A number of GPCRs have been shown to activate MAPK via transactivation of the EGF receptor. This conclusion is based on a series of reports demonstrating that pharmacological inhibitors of the EGF receptor kinase, or expression of dominant negative EGF receptor mutants, prevent the activation of MAPK in response to GPCR ligands such as endothelin-1, lysophosphatidic acid, α -thrombin, angiotensin II, UTP and carbachol [23–28,33]. In most reports to date, the EGF receptor-dependent pathway is the principal route connecting GPCRs with MAPK, and inhibition of the EGF receptor activity alone is enough to interrupt the stimulation of MAPK by the GPCR ligand. In the present study, in contrast, the EGF receptor-dependent component appeared to account for only a part of the response.

Many studies of EGF receptor transactivation by GPCRs report that tyrosine phosphorylation of adapter proteins such as Shc, Cbl and Gab1 is increased by GPCR activation [23,24,27,33,40]. Consistent with these reports, stimulation of m3 receptors increased tyrosine phosphorylation of p52 Shc by 3.6-fold. The increase was blocked by the EGF receptor inhibitor AG1478, and by the Src inhibitor PP1, suggesting that Shc phosphorylation was a result of an interaction with the activated EGF receptor, and with Src. Moreover, since PP1 inhibited the association of the tyrosine-phosphorylated EGF receptor with Shc, without decreasing total EGF receptor tyrosine phosphorylation (Figure 5), it is likely that PP1 does not directly

inhibit EGF receptor tyrosine kinase activity. Similar results have been reported by others [21,24], leading to the suggestion that Src may act 'downstream' of the EGF receptor to mediate tyrosine phosphorylation of Shc, possibly after the latter associates with the activated EGF receptor [34]. The results are also consistent with the possibility that tyrosine-phosphorylated Shc couples the activated EGF receptor to the MAPK cascade, presumably via recruitment of the Grb2–mSos complex [49], although the authors do not rule out the involvement of other adapter proteins in the response.

In a subset of experiments in which the response was assessed, it was noted that tyrosine phosphorylation of p52 Shc induced by carbachol was actually increased in the presence of GF109203X (Figure 4D). This was accompanied by increased tyrosine phosphorylation of the EGF receptor. These effects tended to occur in experiments in which the inhibitory effect of GF109203X on carbachol-induced MAPK activation was less than average. It is important to note that, regardless of the extent of MAPK inhibition by GF109203X, the residual, PKC-independent response was virtually abolished by AG1478 or by PP1. The results suggest that, under certain conditions that are as yet ill-defined, activation of PKC by carbachol may inhibit signalling by the EGF receptor/Src-dependent pathway. In such an instance, addition of the PKC inhibitor GF109203X unmasks, or potentiates, this secondary pathway. This hypothesis is supported by the observation that carbachol inhibited the association of tyrosine-phosphorylated EGF receptor with Shc caused by exogenous EGF, an effect that was reversed by GF109203X (Figure 6B). A similar interaction between carbachol and EGF has also been described by others [50]. The combined effect of carbachol and EGF on MAPK activation did not exceed the response to EGF alone, a result that also supports the notion that carbachol inhibits signalling by EGF (although it is also possible that the magnitude of the MAPK response is restricted by the limited availability of additional factors). A similar PKC-dependent suppression of the EGF receptor transactivation pathway was observed in GN4 rat liver epithelial cells stimulated with angiotensin II [28]. In contrast to these findings, tyrosine phosphorylation of endogenous EGF receptors by carbachol in m1 receptor-expressing HEK cells was reported to be mediated by PKC, as it was inhibited by GF109203X [40]. It is therefore possible that m1 and m3 receptors differentially modulate EGF receptor signalling pathways.

The mechanism linking activated GPCRs to the EGF receptor is unclear. Although EGF receptor transactivation by m1 or angiotensin-II receptors occurred in the absence of detectable EGF release from the cells [26,40], it was recently reported that the GPCR ligands endothelin, carbachol and lysophosphatidic acid activate the EGF receptor via metalloprotease-dependent cleavage of the transmembrane precursor of heparin-binding EGF-like growth factor [51]. It remains to be determined whether this mechanism accounts for the transactivation of the EGF receptor observed in the present study.

In summary, the results indicate that muscarinic m3 receptors regulate the activation of MAPK by two mechanisms; one dependent on PKC, and the other on sequential activation of the EGF receptor and Src tyrosine kinase. The consequences of this dual regulatory mechanism for cell signalling are currently unknown. In PC12 cells, nerve growth factor results in sustained activation of MAPK activity, and cell differentiation, whereas EGF produces a short-lived increase in MAPK, and cell proliferation ([52] and references therein). Moreover, the sustained, but not the early phase of, integrin-mediated MAPK activation is dependent on PKC [53]. It is therefore plausible to propose that the duration of MAPK activation, and therefore the

downstream consequences of m3 receptor stimulation, may depend on whether or not the response is mediated principally by PKC or by transactivation of the EGF receptor, or by a combination of both signals. The existence of multiple, independently regulated pathways linking GPCRs to MAPK may thus expand the repertoire of responses available to a cell exposed to a given extracellular signal.

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