The m1 muscarinic acetylcholine receptor transactivates the EGF receptor to modulate ion channel activity

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Intracellular tyrosine kinases link the G protein-coupled m1 muscarinic acetylcholine receptor (mAChR) to multiple cellular responses. However, the mechanisms by which m1 mAChRs stimulate tyrosine kinase activity and the identity of the kinases within particular signaling pathways remain largely unknown. We show that the epidermal growth factor receptor (EGFR), a single transmembrane receptor tyrosine kinase, becomes catalytically active and dimerized through an m1 mAChR-regulated pathway that requires protein kinase C, but is independent of EGF. Finally, we demonstrate that transactivation of the EGFR plays a major role in a pathway linking m1 mAChRs to modulation of the Kv1.2 potassium channel. These results demonstrate a ligand-independent mechanism of EGFR transactivation by m1 mAChRs and reveal a novel role for these growth factor receptors in the regulation of ion channels by G protein-coupled receptors.

Keywords: dimerization/growth factor receptors/G proteins/neurotransmitter receptors/potassium channels

Introduction

Tyrosine kinases are important components of signaling pathways that couple cell surface receptors to the regulation of many cellular activities including gene expression, proliferation, synaptic organization and ion channel modulation. Although the importance of receptor tyrosine kinases (RTKs) in growth factor signaling has been recognized for some time (Chen *et al.*, 1987), more recent studies have shown that other classes of receptors, including cytokine, integrin, antigen and G protein-coupled receptors (GPCRs), also utilize tyrosine kinases to transduce intracellular signals (Zachary and Rozengurt, 1992; Aoki *et al.*, 1994; August *et al.*, 1994; Chen *et al.*, 1994; Ihle, 1994). Unlike the RTKs, these receptor families lack intrinsic kinase activity and must recruit and activate cytosolic proteins such as the JAK (Stahl and Yancopoulos, 1993; Ihle, 1994), FAK (Zachary and Rozengurt, 1992) and Src family tyrosine kinases (Clark and Brugge, 1993).

Studies of the epidermal growth factor receptor (EGFR) have contributed many insights into the mechanisms by which RTKs transduce proliferative signals. Activation of EGFRs typically begins with ligand-induced receptor

dimerization. The accompanying conformational change results in activation of the cytoplasmic tyrosine kinase domain which in turn leads to autophosphorylation and transphosphorylation of substrates containing Src homology 2, or SH2, domains (reviewed in Ullrich and Schlessinger, 1990). For example, the SH2-containing adaptor molecule termed SHC binds to and is phosphorylated by the activated EGFR (Pelicci *et al.*, 1992). The tyrosine-phosphorylated and membrane-localized SHC protein then serves as a binding site for other proteins which ultimately activate the Ras/MAP kinase signaling cascade and other effectors (Honegger *et al.*, 1987; Moolenaar *et al.*, 1988; Buday and Downward, 1993). While the cytokine, integrin and antigen receptor families lack intrinsic kinase activity, agonist binding to these receptors results in activation of associated tyrosine kinases which mimic RTKs to some extent by recruiting and phosphorylating downstream effector proteins (Clark and Brugge, 1993; Weiss and Littman, 1994).

Although much is known about the roles of RTKs in growth factor signaling, considerably less is understood regarding the activation of tyrosine kinases by GPCRs. Upon ligand activation, a GPCR catalyzes the exchange of GDP for GTP on the α subunit of an associated heterotrimeric G protein, which results in the release of two signaling molecules, namely $G_{\alpha GTP}$ and $G_{\beta\gamma}$. Mitogenic signaling through some GPCRs, such as endothelin or thrombin receptors, involves tyrosine phosphorylation of SHC and activation of the Ras signaling cascade. This response may result primarily from the release of $G_{\beta\gamma}$ subunits (Crespo *et al.*, 1994; van Biesen *et al.*, 1995), possibly through the direct activation of a pleckstrin homology (PH) domain-containing or Src family tyrosine kinase (Tsukada *et al.*, 1994; Luttrell *et al.*, 1997). Alternatively, other types of GPCRs may activate intracellular tyrosine kinases and the Ras pathway indirectly by controlling the level of second messenger molecules (Zachary *et al.*, 1992; Taniguchi *et al.*, 1993; Chen *et al.*, 1994; Lev *et al.*, 1995; Simonson *et al.*, 1996). Interestingly, there is recent evidence that the EGFR also plays a role in mitogenic signaling through thrombin, endothelin and lysophosphatidic acid receptors (Daub *et al.*, 1996). In this case, pharmacological inactivation of the EGFR kinase activity decreases the ability of these GPCRs to induce transcription of the *c-fos* gene or stimulate DNA synthesis.

The muscarinic acetylcholine receptor (mAChR) family consists of five related subtypes (m1–5) that are highly expressed in the central and peripheral nervous systems. The m1, m3 and m5 subtypes couple to $G_{\alpha q}$ type G proteins to stimulate phospholipase C (PLC) activity and thus cause the activation of protein kinase C (PKC) and the release of Ca^{2+} from intracellular stores (Lechleiter *et al.*, 1990a,b; Berstein *et al.*, 1992). In contrast, m2 and m4 mAChRs mediate the inhibition of adenylyl cyclase

activity and activation of inward rectifier K⁺ channels through G_{βγ} subunits derived from G_{αi} and G_{αo} type G proteins (Peralta *et al.*, 1988; Reuveny *et al.*, 1994; Kunkel and Peralta, 1995). Muscarinic receptors have been implicated in complex processes ranging from learning and memory to several CNS disorders such as Alzheimer's and Parkinson's disease (Nathanson, 1987; Taylor, 1990; Nitsch *et al.*, 1992). At the cellular level, mAChRs stimulate tyrosine kinases to regulate such responses as release of the secreted amyloid precursor protein (Slack *et al.*, 1995), activation of the Ras pathway (Crespo *et al.*, 1994) and modulation of delayed rectifier K⁺ channel activity (Huang *et al.*, 1993). However, as is true for several other classes of GPCRs, the mechanisms by which mAChRs stimulate particular tyrosine kinases are not well defined.

Through studies in stable transfected human 293 cells, we have found that the EGFR becomes tyrosine phosphorylated as a result of m1 mAChR signaling. In addition, we show that m1 mAChR-induced EGFR transactivation occurs through a PKC-dependent, Ca²⁺-insensitive pathway. We also demonstrate through cross-linking studies that m1 mAChR-mediated transactivation results in receptor dimerization via an EGF-independent mechanism. Finally, we conducted electrophysiological experiments that reveal a novel function of the m1 mAChR-stimulated EGFR transactivation pathway, namely modulation of the voltage-gated K⁺ channel termed Kv1.2 (Paulmichl *et al.*, 1991; Huang *et al.*, 1993; Morielli *et al.*, 1995). Taken together, this study identifies the EGFR as a target of m1 mAChR signaling and demonstrates a new role for the EGFR in neurotransmitter receptor signaling.

Results

EGFR activation by the m1 mAChR

To assess the ability of m1 mAChRs to stimulate tyrosine kinase activity, we treated stable transfected 293 cells with carbachol, a stable muscarinic agonist, and analyzed cellular lysates by immunoblotting with an anti-phosphotyrosine monoclonal antibody (4G10). Carbachol treatment resulted in a clear increase in the tyrosine phosphorylation of several proteins ranging in mol. wt from 45 to 175 kDa (Figure 1). Treatment with atropine, a muscarinic antagonist, completely blocked this effect. The striking induction in the phosphotyrosine state of multiple proteins following carbachol treatment strongly suggests that one or more tyrosine kinases are activated by m1 mAChRs, although alterations in tyrosine phosphatase activity are also possible.

Since the SHC adaptor protein is an important component of the pathways linking RTKs and some GPCRs to activation of the Ras cascade (Crespo *et al.*, 1994; Pawson, 1995; van Biesen *et al.*, 1995), we next examined whether the p52 isoform of SHC becomes tyrosine phosphorylated in our system. We immunoprecipitated the SHC protein from cells that had been treated with EGF (to activate endogenous EGFRs) or carbachol and analyzed the immunoprecipitates using the 4G10 antibody. As expected, the control treatment with EGF caused a robust increase in the phosphotyrosine content of p52 SHC (Figure 2A). Similarly, carbachol evoked a potent increase in tyrosine-phosphorylated SHC (Figure 2A). Interestingly,

Fig. 1. m1 mAChRs stimulate cellular tyrosine kinase activity. Stable transfected human 293 cells expressing the m1 mAChR were serum starved for 24 h. Unstimulated cells (control), cells stimulated with 1 mM carbachol for 5 min (carb), cells treated with 100 μ M atropine (a mAChR antagonist) and 1 mM carb for 5 min (atr/carb) and cells stimulated with 10% fetal bovine serum (as a positive control) for 5 min (FBS) were lysed. One hundred μ g of protein from each sample were resolved by 10% SDS-PAGE, transferred to nitrocellulose and immunoblotted with an anti-phosphotyrosine monoclonal antibody (4G10-HRP). Bars indicate the positions of the molecular weight markers. Similar results were obtained in at least three experiments.

we also observed a higher molecular weight band of ~175 kDa that co-immunoprecipitated with SHC and became tyrosine phosphorylated in response to either EGF or carbachol treatment. Since p52 SHC was identified originally as a substrate for the 175 kDa EGFR, we re-probed the same nitrocellulose filter with an anti-EGFR antibody and found that the 175 kDa band became labeled (Figure 2A). Thus, activation of the m1 mAChR induces tyrosine phosphorylation of the EGFR. In addition, pre-treatment of cells with a specific inhibitor of the EGFR kinase activity, tyrophostin AG1478, blocked both the EGF- and carbachol-stimulated tyrosine phosphorylation of SHC, as well as the SHC-associated 175 kDa protein identified as the EGFR (Figure 2A; see below).

To explore the specificity of this transactivation pathway, we tested whether another endogenous RTK, the insulin receptor, also becomes tyrosine phosphorylated via m1 mAChR signaling. To this end, we analyzed the phosphotyrosine content of immunoprecipitated insulin receptors before and after carbachol treatment. This analysis demonstrated that carbachol does not induce an increase in the tyrosine phosphorylation of insulin receptors and suggests that the m1 mAChR transactivation pathway exhibits some degree of specificity among different RTKs (Figure 2B).

To establish further that the carbachol-induced increase in the phosphotyrosine content of EGFRs reflects activation of the intrinsic kinase domain, we conducted two types of experiments. First, we tested whether tyrophostin AG1478 could affect the ability of m1 mAChRs to stimulate tyrosine phosphorylation of the EGFR. Previous reports indicate that tyrophostin AG1478 acts as a specific inhibitor of EGFR kinase activity, and has no significant effect on other tyrosine kinases tested (Levitzi and Gazit, 1995). To determine the efficacy and specificity of AG1478 in our system, we analyzed the effect of the drug on EGF- or carbachol-induced tyrosine phosphorylation of

Fig. 2. m1 mAChR-induced tyrosine phosphorylation of the EGFR and SHC. **(A)** Human 293 cells expressing the m1 mAChR were serum starved for 24 h. Unstimulated cells (control), cells stimulated with 10 ng/ml EGF for 15 min (EGF), cells pre-treated with 250 nM AG1478 for 20 min and challenged with 10 ng/ml EGF for 15 min (AG1478/EGF), cells stimulated with 1 mM carbachol for 15 min (carb) and cells pre-treated with 250 nM AG1478 for 20 min and challenged with 1 mM carbachol for 15 min (AG1478/carb) were lysed. Approximately 1 mg of protein was incubated with 1 μ g of anti-SHC antibody. The immunoprecipitates were resolved by 10% SDS-PAGE, transferred to nitrocellulose and immunoblotted with either an anti-phosphotyrosine antibody (top panel), anti-EGFR antibody (middle panel) or anti-SHC antibody (bottom panel). Arrows indicate the positions of p175 EGFR and p52 SHC. **(B)** Human 293 cells expressing the m1 mAChR were serum starved for 24 h. Unstimulated cells (control), cells stimulated with 1 μ M insulin for 1 min (Ins, 1 min), cells stimulated with 1 μ M insulin for 15 min (Ins, 15 min), cells stimulated with 1 mM carbachol for 1 min (carb, 1 min) and cells treated with 1 mM carbachol for 15 min (carb, 15 min) were lysed. Approximately 1 mg of protein from each sample was used in an immunoprecipitation reaction with 2 μ g of anti-insulin receptor (β subunit) antibody. Immunoprecipitates were resolved by 7.5% SDS-PAGE, transferred to nitrocellulose and immunoblotted with either an anti-phosphotyrosine antibody (top panel) or an anti-insulin receptor antibody (β subunit; bottom panel). Arrows indicate the position of the p90 insulin receptor (β subunit). The results shown are representative of at least three experiments.

the EGFR. The phosphotyrosine content of EGFRs immunoprecipitated from cells pre-treated with 250 nM AG1478 prior to EGF or carbachol addition was significantly less than from cells exposed to EGF or carbachol alone (Figure 3A). To confirm the selectivity of AG1478 in our system, we also tested whether the carbachol-dependent activation of two distinct types of cytoplasmic tyrosine kinases, p60 Src and p125 FAK, or activation of insulin receptors by insulin, was affected by the drug. These experiments demonstrated that cells pre-treated with 1 μ M AG1478 failed to block carbachol-induced tyrosine phosphorylation of the Src and FAK kinases, or activation

of the insulin receptor by its ligand (Figure 3B). Therefore, experiments with AG1478 indicate that the carbachol-induced increase in tyrosine phosphorylation most likely results from activation of the EGFR's intrinsic kinase domain, rather than through a transphosphorylation mechanism involving a distinct tyrosine kinase. To test this interpretation, we also conducted immunoblotting experiments using a monoclonal antibody that recognizes specifically the kinase-activated conformation of the EGFR (Emlet *et al.*, 1997). Cells were treated with carbachol or EGF and the lysates were immunoblotted and probed with the activation-specific antibody (Figure 3C). This antibody clearly detected the EGFRs obtained from carbachol- and EGF-treated cells. Taken together, these findings establish that the m1 mAChR-mediated pathway results in activation of the intrinsic kinase domain of the EGFR.

EGF-independent EGFR dimerization

Since previous studies have shown that dimerization of EGFRs is a prerequisite for EGF-induced activation, we asked whether the m1 mAChR-induced transactivation pathway also involves the formation of EGFR dimers. The compound bis(sulfosuccinimidyl)suberate (BS₃) is an effective EGFR cross-linking reagent in intact living cells (Sorkin and Carpenter, 1991). To determine if EGFR dimers form in response to m1 mAChR activation, BS₃ was added to 293 cells following carbachol or EGF treatment; the cross-linking reactions were then terminated by the addition of a basic glycine solution. Immunoprecipitation of the EGFR followed by immunoblot analysis revealed that EGFR dimers formed in response to either EGF or carbachol treatment (Figure 4A). The extent of EGFR dimer formation evoked by each agonist appeared comparable, although the degree to which these agonists induced EGFR phosphorylation and activation was different (see Figures 2 and 3). It is possible that this discrepancy may be due to the efficiency of the cross-linking reaction. For example, if EGF induced a somewhat greater proportion of receptor dimers compared with carbachol, inefficiency in the cross-linking reaction may have precluded such detection. However, these findings do demonstrate that transactivation of the EGFR via the m1 mAChR is coincident with EGFR dimerization.

It is possible that carbachol binding to the m1 mAChR triggers a signaling pathway that somehow induces the release of EGF which then binds, dimerizes and activates the EGFR through an autocrine mechanism. Therefore, we investigated whether carbachol induced the release of EGF from these cells. Culture supernatants from EGF- or carbachol-treated cells were collected, concentrated and subjected to immunoblot analysis with an anti-EGF antibody. We previously found that 20 ng of EGF was required to stimulate the phosphotyrosine content of EGFRs significantly under the conditions used for all experiments described in this study (not shown). However, we were unable to detect the presence of EGF in the supernatants collected from carbachol-treated cells even though the anti-EGF antibody was able to detect ≤ 10 ng of EGF in this assay (Figure 4B). Therefore, we conclude that the m1 mAChR probably does not induce EGFR dimerization through an autocrine mechanism.

EGFR transactivation is a PKC-dependent, Ca^{2+} -insensitive response

Activation of PLC β by m1 mAChRs leads to the activation of the serine/threonine kinase family termed protein kinase C (PKC) and the release of Ca^{2+} from intracellular stores (Berridge, 1993). To test the role of PKC in the m1 mAChR-mediated transactivation pathway, we treated 293 cells with the PKC activator, phorbol 12-myristate 13-acetate (PMA). This treatment dramatically increased the phosphotyrosine content of immunoprecipitated EGFRs (Figure 5). Therefore, activation of PKC is sufficient to cause tyrosine phosphorylation of the EGFR. However,

to determine if PKC lies in the pathway between m1 mAChR stimulation and EGFR transactivation, we treated cells with the specific PKC inhibitor, GF109203X (Toullec *et al.*, 1991), for 20 min prior to addition of carbachol. Treatment with GF109203X potently inhibited the subsequent carbachol-induced tyrosine phosphorylation of the EGFR in these cells (Figure 5). Thus, PKC activation appears to be both sufficient and necessary for m1 mAChR-induced EGFR activation.

Since carbachol induces a large increase in cytoplasmic Ca^{2+} in 293 cells expressing m1 mAChRs, we asked whether elevated Ca^{2+} also contributes to the transactivation of EGFRs. To this end, we applied the Ca^{2+} ionophore, A23187 (1 μ M, 10 min), and again analyzed the phosphotyrosine content of the immunoprecipitated EGFR. A23187 treatment did not induce an increase in the phosphotyrosine content of EGFRs (Figure 5) even though direct Ca^{2+} imaging analysis of these cells confirmed that the ionophore treatment evoked a rapid and dramatic elevation in intracellular Ca^{2+} (not shown). Consistent with this result, cells pre-treated with the Ca^{2+} chelators EGTA and EDTA prior to carbachol treatment also responded normally (Figure 5; see Materials and methods for additional details). Therefore, increases in intracellular Ca^{2+} concentration due to Ca^{2+} influx do not appear to be an important signal for the m1 mAChR-induced EGFR transactivation mechanism.

Fig. 3. m1 mAChR-induced EGFR tyrosine phosphorylation requires catalytic activation of the EGFR. (A) Human 293 cells expressing the m1 mAChR were serum starved for 12 h. Unstimulated cells (control), cells stimulated with 10 ng/ml EGF for 15 min (EGF), cells stimulated with 1 mM carbachol for 15 min (carb), cells pre-treated with 250 nM AG1478 for 20 min and challenged with 10 ng/ml EGF for 15 min (AG1478/EGF) and cells pre-treated with 250 nM AG1478 for 20 min and challenged with 1 mM carbachol for 15 min (AG1478/carb) were lysed. Approximately 1 mg of protein was immunoprecipitated using 2 μ g of anti-EGFR antibody. Immunoprecipitates were resolved by 6.5% SDS-PAGE, transferred to nitrocellulose and immunoblotted with either an anti-phosphotyrosine antibody (top panel) or anti-EGFR antibody (bottom panel). The arrows indicate the position of p175 EGFR. (B) Human 293 cells expressing the m1 mAChR were serum starved for 12 h. To determine whether AG1478 affects the ability of the m1 mAChR to activate two types of intracellular tyrosine kinases, untreated cells (control), carbachol-treated cells (carb) and cells treated with 1 μ M AG1478 plus carbachol (AG1478/carb) were lysed (top panels). Approximately 1 mg of protein from each sample was immunoprecipitated using 1 μ g of either anti-FAK antibody or anti-Src antibody. The resulting immunoprecipitates were then immunoblotted with the anti-phosphotyrosine, anti-FAK or anti-Src antibodies as indicated. Similar experiments were conducted to test whether AG1478 affects the ability of insulin to activate its cognate receptor (bottom panels). Unstimulated cells (control), cells stimulated with 1 μ M insulin for 10 min (Ins) and cells treated with 1 μ M AG1478 plus 1 μ M insulin (AG1478/Ins) were lysed and immunoprecipitated with an anti-insulin receptor (β subunit) antibody. The samples were immunoblotted and probed with the anti-phosphotyrosine or anti-insulin receptor (β subunit) antibodies as indicated. (C) Human 293 cells expressing the m1 mAChR were serum starved for 12 h. Unstimulated cells (control), cells stimulated with 10 ng/ml EGF for 15 min (EGF) and cells stimulated with 1 mM carbachol for 15 min (carb) were lysed. Approximately 100 μ g of protein from whole cell lysates were resolved by 7% SDS-PAGE, transferred to nitrocellulose and immunoblotted with either an EGFR antibody that recognizes the active conformation of the receptor (EGFR 'act') or the standard EGFR antibody used throughout this study (EGFR). Arrows indicate the position of p175 EGFR. The results shown are representative of at least three experiments.

Fig. 4. EGF-independent EGFR dimerization. (A) Human 293 cells expressing the m1 mAChR were serum starved for 20 h. Unstimulated cells (control), cells stimulated with 100 ng/ml EGF for 15 min (EGF) and cells stimulated with 1 mM carbachol for 15 min (carb) were cross-linked with 3 mM bis(sulfosuccinimidyl) suberate for 20 min followed by addition of 250 mM glycine for 5 min. Cells were then lysed and immunoprecipitated with 2 μ g of anti-EGFR antibody. Immunoprecipitates were resolved by 6% SDS-PAGE, transferred to nitrocellulose and immunoblotted with an anti-EGFR antibody. The arrows indicate the position of the EGFR monomer and dimer. (B) Human 293 cells expressing the m1 mAChR were serum starved for 20 h. Culture supernatants from unstimulated cells (control), cells stimulated with 50 ng/ml EGF for 15 min (EGF) and cells stimulated with 1 mM carbachol for 15 min (carb) were concentrated. To detect EGF in the culture supernatants, samples were resolved by 12% SDS-PAGE, transferred to nitrocellulose and immunoblotted with an anti-EGF monoclonal antibody (top panel). The sensitivity of the anti-EGF antibody was tested by blotting defined amounts of EGF (bottom panel).

Transactivated EGFRs contribute to K⁺ channel modulation

Activation of the EGFR typically is associated with mitogenic responses (Ullrich and Schlessinger, 1990; Daub *et al.*, 1996). However, previous research in 293 cells has shown that m1 mAChRs cause a suppression of the ionic current carried by the delayed rectifier K⁺ channel, termed Kv1.2, through a tyrosine kinase-dependent mechanism (Huang *et al.*, 1993; Lev *et al.*, 1995). Since the experiments described above suggest that transactivated EGFRs may account for a significant fraction of m1 mAChR-stimulated tyrosine kinase activity in these cells, we tested the possibility that the transactivated receptors may contribute to modulation of the Kv1.2 channel. To test this idea, we again used the specific EGFR kinase inhibitor tyrophan AG1478 with the expectation that if m1 mAChR-induced channel suppression involves EGFR transactivation, then such suppression should be blocked at least in part by pre-treatment with AG1478. Prior to any treatment, Kv1.2 channels expressed in 293 cells generated an outward K⁺ current typical of delayed rectifiers (Figure 6A, left panel). This channel activity

Fig. 5. The m1 mAChR transactivates the EGFR through a PKC-dependent, Ca²⁺-insensitive pathway. Human 293 cells expressing the m1 mAChR were serum starved for 14 h. Unstimulated cells (control), cells stimulated with 1 mM carbachol for 15 min (carb), cells stimulated with 10 μ M phorbol 12-myristate 13-acetate for 10 min (PMA), cells stimulated with 10 μ M A23187 for 10 min (A23187), cells stimulated with 10 μ M PMA and 10 μ M A23187 for 10 min (PMA + A23187), cells pre-treated with 1 mM EDTA and 1 mM EGTA for 10 min and challenged with 1 mM carbachol for 15 min (EDTA + EGTA/carb) and cells pre-treated with 15 μ M GF109203X (a protein kinase C inhibitor) for 30 min and challenged with 1 mM carbachol for 15 min (GF109203X/carb) were lysed. Approximately 1 mg of protein from each sample was immunoprecipitated using 2 μ g of anti-EGFR antibody. Immunoprecipitates were resolved by 6.5% SDS-PAGE, transferred to nitrocellulose and immunoblotted with either the anti-phosphotyrosine antibody (top panel) or anti-EGFR antibody (bottom panel). An arrow indicates the position of p175 EGFR. Results are representative of at least three experiments.

became strongly suppressed upon carbachol treatment (Figure 6A, right panel, and C). Importantly, carbachol-induced suppression of the Kv1.2 current was blocked substantially by pre-treatment with AG1478 (Figure 6B, right panel, and C). If the AG1478-dependent reduction in the ability of m1 mAChRs to modulate the channel resulted from inhibition of EGFR kinase activity, then these results predict that direct activation of EGFRs with EGF should also cause an AG1478-sensitive suppression of Kv1.2 activity. Consistent with this interpretation, application of EGF caused a strong suppression of the Kv1.2 current in these cells (Figure 6D). Moreover, the EGF-induced response was blocked completely by pre-treatment with AG1478 (Figure 6D). Therefore, these electrophysiological experiments provide the first demonstration that transactivated EGFRs are an important component in a pathway linking a G protein-coupled receptor to modulation of a defined ion channel.

Discussion

The importance of RTKs in growth factor signaling and oncogenic transformation has been clear for some time. However, only recently have tyrosine kinases been recognized as important components of particular GPCR signaling pathways (Huang *et al.*, 1993; Crespo *et al.*, 1994; van Biesen *et al.*, 1995; Chen *et al.*, 1996). The studies described here demonstrate that transactivated EGFRs can play an important role in m1 mAChR-mediated signaling both at the membrane and within the cell. In particular, these studies indicate a novel role for transactivated EGFRs in the modulation of ion channel activity by GPCRs.

Fig. 6. EGFR transactivation contributes to m1 mAChR-mediated suppression of the Kv1.2 potassium channel. **(A)** Human 293 cells expressing the m1 mAChR and Kv1.2 channel were assayed using whole cell voltage clamp analysis. A family of whole cell currents were elicited by voltage steps from -80 to $+60$ mV from a holding potential of -60 mV. Each trace is the average of currents elicited from 10 individual cells. Currents generated before (left panel) and 10 min after (right panel) application of 1 mM carbachol to the bath in the absence of AG1478. **(B)** Currents elicited before (left panel) and 10 min after (right panel) application of 1 mM carbachol to cells which had been pre-treated for 10 min with AG1478. **(C)** Quantification of the currents shown in (A) and (B). The mean steady-state current elicited by a pulse to 0 mV from a holding potential of -60 mV was significantly reduced after application of carbachol ($P \leq 0.01$, t test). Prior treatment with AG1478 strongly blocked such suppression ($n = 10$ for each condition). Error bars represent the standard error of the mean. **(D)** EGF significantly suppressed the mean steady-state amplitude of Kv1.2 current generated as in (C) ($P \leq 0.01$, t test) and such suppression is fully blocked by AG1478 ($n = 10$ for each condition). The amplitude and error bars are as in (C).

The results of biochemical and pharmacological experiments provide several insights into the mechanism of EGFR transactivation by m1 mAChRs. Immunoprecipitation studies revealed that the abundance of tyrosine-phosphorylated EGFR was increased significantly by carbachol treatment. Since the EGFR kinase inhibitor AG1478 blocked both EGF- and carbachol-induced increases in EGFR phosphotyrosine content, we conclude that the carbachol-induced increase also represents EGFR autophosphorylation via activation of its intrinsic tyrosine kinase domain. Activation of the EGFR was also supported by immunoblotting analysis with an antibody that specifically recognized the active conformation of EGFRs isolated from carbachol-treated cells. Previous studies have shown that stimulation of the tyrosine kinase domain of the EGFR follows a second order reaction rate constant with respect to the EGFR concentration, indicating that dimerization of monomeric EGFRs is essential for full activation by EGF (reviewed in Schlessinger, 1988; Ullrich and Schlessinger, 1990; Canals, 1992). Our cross-linking results present the first demonstration that EGFR dimers are also formed as a consequence of m1 mAChR activation. Furthermore, we were unable to detect the presence of EGF in carbachol-treated culture supernatants, indicating that carbachol does not induce an autocrine release of EGF. Thus, carbachol binding to the m1 mAChR generates an intracellular signaling cascade that leads to EGF-independent receptor dimerization and catalytic activation.

We conducted several experiments to investigate which intracellular signals may play a role in the EGFR transactivation pathway. Since m1 mAChRs are strong activators of PLC β , we tested the importance of PKC and intracellular Ca^{2+} release in this process. Our data indicate that only one of the two PLC signaling branches, namely

PKC, participates in the transactivation mechanism. Activation of PKC by phorbol esters caused a strong increase in the abundance of tyrosine-phosphorylated EGFRs, while application of a PKC inhibitor, GF109203X, completely blocked EGFR activation by carbachol. In contrast, EGFR transactivation does not require Ca^{2+} -dependent signals, as demonstrated by the inability of the Ca^{2+} ionophore, A23187, to induce EGFR phosphorylation or Ca^{2+} chelators to block carbachol-mediated transactivation. There is recent evidence in other cell types that Ca^{2+} influx through voltage-gated channels can be sufficient to cause tyrosine phosphorylation of the EGFR (Rosen and Greenberg, 1996). However, we find that transactivation by the m1 mAChR in human 293 cells involves primarily PKC-dependent signals. It is possible that PKC directly phosphorylates the EGFR, although numerous studies have shown that such phosphorylation is associated with down-regulation of EGF-dependent receptor activation (Cochet *et al.*, 1984; Downward *et al.*, 1985; Fearn and King, 1985). Alternatively, PKC-stimulated signals may lead to the activation of intracellular tyrosine kinases which in turn phosphorylate the EGFR. We note that the ability of m1 mAChRs to activate the Src and FAK tyrosine kinases in the absence of carbachol-induced EGFR tyrosine phosphorylation (i.e. following AG1478 treatment) indicates that neither of these tyrosine kinases directly phosphorylate the EGFR in our system.

Recent crystal structures of the cytoplasmic tyrosine kinase domains of the insulin and fibroblast growth factor receptors have provided important information regarding the mechanism by which these RTKs are activated (Hubbard *et al.*, 1994; Mohammadi *et al.*, 1996). While similar structural information is not yet available for the EGFR, numerous biochemical studies have shown that

dimerization of the monomeric receptor by EGF must occur in order to induce its tyrosine kinase activity (Yarden and Schlessinger, 1987; Ullrich and Schlessinger, 1990; Schlessinger and Ullrich, 1992). Our findings provide evidence that EGFR dimerization and activation can occur in the absence of EGF. Whether EGF-independent dimerization of the receptor is responsible for kinase activation, or vice versa, remains unknown. It is possible that carbachol regulates an intracellular signaling pathway that leads to an alteration in proteins that may associate with the EGFR or perhaps cause a covalent modification of the EGFR cytoplasmic domain. This may induce a conformational change which in turn causes dimerization of the EGFR resulting in kinase activation. Alternatively, m1 mAChR-induced dimerization of the EGFR may not be essential for activation, but simply be a secondary effect. For example, m1 mAChR-generated signals may cause conformational changes in the EGFR monomer that lead directly to kinase activation. In this case, dimerization of the EGFR may occur subsequent to kinase activation. An intracellular mechanism of inducing EGFR dimerization would probably require interaction between the EGFR cytoplasmic domains to provide sufficient support for stable dimer formation. Although the EGFR extracellular domain is normally responsible for stabilizing EGF-induced dimers (Hurwitz *et al.*, 1991; Lax *et al.*, 1991), recent homology modeling studies suggest that the cytoplasmic EGFR kinase domain may also form dimers and higher aggregates (Murali *et al.*, 1996). A full kinetic analysis of the carbachol-induced EGFR dimerization and activation steps may allow us to distinguish between these two possibilities.

Previous studies have shown that m1 mAChRs suppress the ionic currents generated by the Kv1.2 potassium channel through a pathway involving PLC-stimulated tyrosine kinase activity (Huang *et al.*, 1993; Morielli *et al.*, 1995). Because of the selective nature of the EGFR kinase inhibitor AG1478 (Levitski and Gavit, 1995), we were able to test the possibility that modulation of this K⁺ channel may involve transactivation of the EGFR. Electrophysiological studies demonstrated that AG1478 treatment does block a large portion of the carbachol-induced channel suppression, indicating that transactivated EGFRs are a significant component in a pathway by which m1 mAChRs regulate the Kv1.2 channel. We note, however, that the transactivation pathway is not entirely responsible for m1 mAChR-mediated channel modulation, since AG1478 does not completely block the entire carbachol response. Consistent with this observation, Ca²⁺-activated tyrosine kinases can also cause Kv1.2 suppression, indicating that there are multiple pathways to regulate the same ion channel (Huang *et al.*, 1993; Morielli *et al.*, 1995). For example, the FAK-related kinase termed PYK2 has been shown to be Ca²⁺ activated and capable of mediating suppression of the Kv1.2 current (Lev *et al.*, 1995). In conclusion, our studies reveal a novel role for EGFRs in the regulation of membrane excitability and intracellular signaling through m1 mAChRs and perhaps other G protein-coupled neurotransmitter receptors.

Materials and methods

Reagents

Antibodies were purchased from the following vendors: Upstate Biotechnology (4G10-HRP, anti-EGFR, anti-EGF, anti-SHC and anti- β subunit

insulin receptor); Santa Cruz (anti-Src); and Transduction Laboratories (anti-FAK and activation-specific anti-EGFR). Protein G and protein A-Sepharose beads were purchased from Sigma and Pharmacia, respectively. The protein kinase C activator, PMA, and the Ca²⁺ ionophore, A23187, were purchased from Sigma. The protein kinase C inhibitor, GF109203X, was purchased from Research Biochemical International. The EGFR kinase inhibitor, tyrophostin AG1478, was purchased from Calbiochem. The EGFR cross-linking reagent, BS₃, was purchased from Pierce.

Cell culture

Human embryonic kidney 293 cells were transfected by the calcium phosphate method. Stable cell lines expressing both the m1 mAChR and Kv1.2 proteins were obtained as described by Peralta *et al.* (1988). SHC, EGFRs, insulin receptors, Src and FAK proteins are endogenously expressed by these cells. The 293 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and streptomycin, 2 mM L-glutamine, and selected with 500 μ g/ml of G418. For intracellular Ca²⁺ measurements, the cells were loaded with fura-2 AM for 1 h in the same medium and changes in Ca²⁺ concentration were determined by ratiometric analysis as described previously (Dell'Acqua *et al.*, 1993). Addition of 1 mM EDTA and EGTA to the media blocked carbachol-induced Ca²⁺ influx.

Immunoprecipitation and immunoblot analysis

293 cells stably expressing the m1 receptor were grown to confluency in 60 mm dishes. Cells subsequently were serum starved in DMEM containing 0.1% FBS for 14–22 h and treated with the appropriate ligand and/or drug for varying lengths of time as described in the figure legends. Cells were lysed in a modified RIPA buffer containing Tris-HCl (50 mM, pH 7.4), 1% NP-40, sodium deoxycholate (0.25%), sodium chloride (150 mM), EGTA (1 mM), phenylmethylsulfonyl fluoride (PMSF; 1 mM), leupeptin (10 μ g/ml), aprotinin (10 μ g/ml), sodium orthovanadate (1 mM) and sodium fluoride (1 mM). After incubating for 30 min at 4°C, the cell lysates were centrifuged at 12 000 g for 10 min. and the supernatant was collected. In determining cellular phosphotyrosine proteins (Figure 1), ~100 μ g of proteins as estimated by the Bradford assay were resolved by 10% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with 4G10-HRP. For immunoprecipitation experiments, ~750 μ g to 1 mg of protein were pre-cleared with either protein G-agarose or protein A-Sepharose beads for 30 min at 4°C. Then 1–2 μ g of the appropriate antibody were added to the pre-cleared samples and incubated for 6 h at 4°C followed by the addition of either protein G-agarose or protein A-Sepharose beads for 2 h at 4°C. Antibody-antigen complexes were washed three times with a buffer containing Tris-HCl (50 mM, pH 7.4), EDTA (1 mM), EGTA (1 mM), and boiled in SDS sample buffer. The immunoprecipitates were resolved by SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted with the desired antibody. When appropriate, the nitrocellulose membranes were stripped and reprobed with another antibody.

EGFR cross-linking

293 cells expressing the m1 mAChR were grown to confluency in a 60 mm dish. Subsequently, cells were serum starved in DMEM containing 0.1% FBS for 14 h and treated with either EGF (1 μ g/ml) for 15 min at 37°C or carbachol (1 mM) for 15 min at 37°C. Cells were washed with ice-cold phosphate-buffered saline (PBS) and the cross-linking reagent BS₃ (3 mM) was added and incubated for 20 min at 4°C. To terminate the cross-linking reaction, cells were washed with ice-cold PBS containing glycine (250 mM, pH 9.0) and incubated for 5 min at 4°C. After decanting the glycine solution, cells were washed and solubilized in a buffer containing Triton X-100 (1%), glycerol (10%), HEPES (50 mM, pH 7.4), sodium orthovanadate (1 mM), PMSF (1 mM), leupeptin (10 μ g/ml) and aprotinin (10 μ g/ml). The cell lysates were incubated for 20 min at 4°C and centrifuged at 12 000 g for 10 min to get rid of the insoluble debris. To the supernatant, 2 μ g of anti-EGFR antibody were added (6 h, 4°C) followed by addition of protein G-agarose beads (12 h, 4°C). The immunoprecipitates were resolved by 6% SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-EGFR antibody.

EGF detection

To detect the presence of EGF in the culture media, culture supernatant from serum-starved cells treated with EGF (50 ng/ml, 15 min at 37°C) or carbachol (1 mM, 15 min at 37°C) and untreated cells (control) were collected and concentrated in a Centricon 3 concentrator. The

concentrated samples subsequently were resolved in 12% SDS-PAGE, transferred to nitrocellulose membrane and immunoblotted with an anti-EGF antibody. The sensitivity of the anti-EGF antibody was determined by resolving known amounts of EGF by 6% SDS-PAGE, transferring the samples to nitrocellulose and immunoblotting with the anti-EGF antibody.

Electrophysiology

Whole cell patch-clamp recordings were taken from human embryonic kidney 293 cells stably expressing both m1 mAChRs and Kv1.2 potassium channels. The cells were plated at low density on poly-D-lysine-coated glass coverslips and placed in low serum (0.1%) growth medium for between 24 and 72 h prior to use. All recordings were taken from isolated cells. Data were collected using an Axopatch 200 patch-clamp interfaced to a Gateway 2000 486 computer using pCLAMP V6 (Axon Instruments). Recording pipets (VWR 100 μ l) were pulled to a resistance of 1 M Ω . All traces were low pass filtered at 1 kHz and leak subtracted using a P/8 protocol from a holding potential of -60 mV. The pipet solution contained 60 mM K₂SO₄, 1.2 mM KCl, 5 mM MgSO₄, 5 mM Na⁺-HEPES and 35 mM sucrose (pH 7.1). The external (bath) solution contained 118 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 0.8 mM MgCl₂, 5 mM Na⁺-HEPES and 23 mM glucose (pH 7.4). Tetraethyl ammonium chloride (TEA) was included in the bath (20 mM) to block other endogenous potassium currents. Sodium orthovanadate (1 mM) was included in the bath to suppress endogenous tyrosine phosphatase activity. For receptor-induced suppression of Kv1.2, carbachol (1 mM) or EGF (1 μ M or 240 nM) was added to the bath 10 min after cells had been equilibrated to 37°C. Recordings from multiple individual cells on a single coverslip commenced 10 min after ligand addition and continued for 50 min. To test the effect of the EGFR kinase inhibitor, AG1478 was added to the bath for at least 10 min prior to the application of ligand.

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