Involvement of Ras and Raf in the G\textsubscript{i}-coupled Acetylcholine Muscarinic m2 Receptor Activation of Mitogen-activated Protein (MAP) Kinase Kinase and MAP Kinase*

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Sim Winitz, Marjane Russell, Nan-Xin Qian, Anne Gardiner, Lori Dwyer, and Gary L. Johnson

From the Division of Basic Sciences, National Jewish Center for Immunology and Respiratory Medicine, Denver, Colorado 80206 and the Department of Pharmacology and School of Pharmacy, University of Colorado Medical School, Denver, Colorado 80262

Stimulation of the acetylcholine muscarinic m2 receptor (m2R) expressed in Rat la fibroblasts results in the activation of the cytoplasmic mitogen-activated protein kinase (MAPK). Concomitant with carbachol stimulation of the m2R was the activation of MEK (MAPK kinase) and Raf. MEK is the dual function kinase that phosphorylates and activates MAPK. Raf is a serine/threonine kinase capable of phosphorylating and activating MEK. Carbachol stimulation of the m2R also activated Ras. Pertussis toxin treatment of Rat la cells inhibited the m2R-mediated activation of Ras, Raf, MEK, and MAPK. In contrast, epidermal growth factor receptor-mediated activation of Ras, Raf, MEK, and MAPK was pertussis toxin-insensitive. m2R activation of Ras, Raf, and MAPK was insensitive to inhibition by genistein, while the epidermal growth factor receptor-induced responses were inhibited by genistein. The findings demonstrate that both Ras and Raf can be regulated by seven-membrane-spanning receptors (i.e. thrombin receptor) are capable of stimulating the EGF receptor (17). Both tyrosine kinase-encoded growth factor receptors (i.e. EGF receptor) and G protein-coupled receptors (i.e. thrombin receptor) are capable of stimulating the rapid activation of MAPKs (18).

In order to define G\textsubscript{i}-regulated signal transduction networks leading to MAPK activation, we expressed the G\textsubscript{i}-coupled m2R in Rat la cells. In this report, we demonstrate that acute stimulation of the m2R leads to the activation of Ras and Raf in a G\textsubscript{i}-dependent manner.

MATERIALS AND METHODS

Expression of Muscarinic m2 Receptor in Rat la Cells—Rat la cells were co-transfected with the expression plasmid pCD, containing the cDNA for the muscarinic m2R, and LNCX, which contained the neomycin resistance gene. G418-resistant clones were isolated and seeded into 12-well dishes for screening with the muscarinic radioligand L-[N-methyl-\textsuperscript{3}H]scopolamine methylchloride (NMS). Two positive clones were further characterized by saturation binding analysis and shown to express approximately 5 \times 10\(^{6}\) and 1.4 \times 10\(^{6}\) m2 receptors/cell. Carbachol stimulation of the m2R expressed in each clone resulted in inhibition of chola toxin-stimulated adenylyl cyclase activity (not shown).

MAPK and MEK Assays—MAPK activity was assayed as described previously (19). Immunoblotting of the fractions with anti-MAPK antibody showed that the peaks of activity corresponded with immunoreactive p42 and p44 MAPK. MEK activity was assayed from cell lysates fractionated on a Mono S FPLC column (20).

Ras Activation Assays—Activation of Ras was determined by analyzing the ratio of GTP to GDP bound to immunoprecipitated Ras from control and stimulated Rat la cells (21). Quantitation of radiolabeled GDP and GTP was accomplished using a Molecular Dynamics PhosphoImager.

Raf Assays—To assay Raf activation, recombinant kinase inactive MEK was used as a substrate (20, 22, 23). Stimulated or control cells were lysed and Raf immunoprecipitated with anti-c-Raf antibody (Santa Cruz Biotechnology). Immunoprecipitates were assayed using recombinant kinase inactive MEK (25-50 ng) and [\textsuperscript{32}P]ATP (10 \muCi).

RESULTS

Rat la cells express the pertussis toxin-sensitive G protein \(\alpha\) subunits \(\alpha_4\) and \(\alpha_9\) but not \(\alpha_\gamma\) (not shown). For this reason the m2R was expressed in Rat la cells to promote selective coupling and activation of G\textsubscript{i}-regulated response pathways including the MAPK regulatory network (1, 2, 12). Fig. 1 shows that carbachol treatment of Rat la cells, expressing approximately G proteins found in fibroblasts and many other cell types. The classically defined G\textsubscript{i}-regulated response is inhibition of adenylyl cyclase activity (4). It is clear, however, that G\textsubscript{i} subunits (\(\alpha_i\)) are capable of regulating specific potassium channels (5), may be involved in controlling intra-Golgi vesicular transport (6), and are involved in mitogenic responses to thrombin and lysophosphatidic acid (7, 8). G proteins are expressed at sufficient levels in many cell types that, when activated, the \(\beta\) subunits dissociated from \(\alpha_\gamma\)-GTP can also contribute to the regulation of effectors including specific isoforms of adenylyl cyclase and phospholipase C (9, 10).

Expression of the GTPase-inhibited \(\alpha_4\) polypeptide in Rat la fibroblasts alters their normal growth control and induces a transformed phenotype (11). The transformation of Rat la cells by GTPase-inhibited \(\alpha_4\) can be dissociated from the regulation of adenylyl cyclase, phospholipase C\(\beta\), and specific ion channels (11, 12). It was found with Rat la cells that GTPase-inhibited \(\alpha_4\) polypeptide expression constitutively activated mitogen-activated protein kinases (MAPKs). MAPKs are serine/threonine kinases that phosphorylate and regulate the activity of several proteins including Ras90 (13), cPLA\(_2\) (14, 15), c-Myc (16), and the EGF receptor (17). Both tyrosine kinase-encoded growth factor receptors (i.e. EGF receptor) and G protein-coupled receptors (i.e. thrombin receptor) are capable of stimulating the rapid activation of MAPKs (18).

The acetylcholine muscarinic m2 receptor (m2R)\(^{1}\) couples predominantly to the pertussis toxin-sensitive G proteins G\textsubscript{i} and G\textsubscript{j} (1, 2). G\textsubscript{i} is limited in tissue distribution and is involved in the regulation of specific ion channels (3). In contrast, G\textsubscript{j} proteins are ubiquitously expressed and are the most abundant
1.4 x 10^6 m2R/cell, activated MAPK to levels similar to those observed with stimulation of the EGF receptor. m2R stimulation of MAPK activity was completely inhibited by pretreatment of the cells with pertussis toxin, consistent with the selective coupling of the m2R with G_i proteins, which are pertussis toxin substrates (Fig. 1). Carbachol stimulation of the m2R did not activate phospholipase C activity in Rat la cells (not shown). Similar to previous reports (24), the ability of the EGF receptor to activate MAPK was completely pertussis toxin-insensitive and does not require a functional G_i protein. MEK, the dual function tyrosine and threonine kinase that phosphorylates MAPK (22), was similarly activated by the stimulation of the m2R or the EGF receptor (Fig. 2). MEK activation by the m2R was inhibited by treatment of Rat la cells with pertussis toxin, whereas the EGF receptor regulation of MEK was again pertussis toxin-insensitive (not shown).

The regulation of MEK and MAPK activity in response to tyrosine kinases has been shown to involve the activation of Raf (23). It was unclear, however, if selective G_i-coupled receptors such as the m2R could also regulate Raf activity. Using purified recombinant MEK as a phosphorylation substrate for Raf, it was found that carbachol activated Raf in a manner similar to that observed with EGF (Fig. 3). The m2R-mediated activation of Raf was completely inhibited by pertussis toxin treatment of the cells, but the EGF receptor response was refractory to pertussis toxin. This is the first demonstration that a G_i-coupled seven-membrane-spanning receptor such as the m2R is capable of activating Raf.

An interaction between Raf and Ras has been defined (25), strongly implicating a linkage between Ras activation and Raf activation (26). Fig. 4 demonstrates that carbachol stimulation of the m2R in Rat la cells caused a time-dependent activation of Ras. Ras activation measured by the formation of Ras-GTP

![Graph showing MAPK activity](image)

**Fig. 1.** Muscarinic m2 receptor stimulation of MAPK activity in Rat la cells. A Rat la cell clone expressing 1.4 x 10^6 m2R per cell (clone 61) was serum-starved overnight in medium containing 0.1% bovine serum albumin in the presence or absence of 100 ng/ml pertussis toxin (PTx). Cells were challenged with 100 μM carbachol or 30 ng/ml EGF for 3 min. Carbachol had no effect on MAPK activity in wild-type Rat la cells, and atropine (10 μM) completely blocked the m2R response but had no effect on EGF (not shown). The results are representative of three independent experiments and are similar to that of two independent m2R-expressing Rat la cell clones.

![Graph showing MEK activity](image)

**Fig. 2.** MEK (MAPK kinase) activation in response to carbachol and EGF. Cell extracts were prepared from carbachol and EGF-stimulated, m2R-expressing Rat la cells as described for Fig. 1. A, MEK resolved by Mono S chromatography. The results are representative of three independent experiments. B, Immunoblotting with a rabbit anti-serum recognizing the C terminus of MEK-1 specifically recognized a 45-kDa protein that co-eluted with MEK enzymatic activity.

![Graph showing Raf activation](image)

**Fig. 3.** Raf activation in response to carbachol and EGF. Serum-starved wild-type (WT) Rat la cells and m2R-expressing Rat la cells were stimulated with 100 μM carbachol or 30 ng/ml EGF for 3 min as described in Fig. 1. Cell lysates were prepared and Raf-1 was immunoprecipitated. Control refers to immunoprecipitation in the absence of Raf antibody. Purified recombinant kinase inactive MEK was used as a Raf substrate in an in vitro kinase assay in the presence of γ-[32P]ATP (10 μCi). After incubation for 10 min, the reaction was stopped with Laemmli SDS sample buffer and the proteins resolved by SDS-polyacrylamide gel electrophoresis (10% acrylamide). Purified recombinant wild-type (WT) MEK was autophosphorylated and used as a standard. A, autoradiograph of phosphoproteins from Raf immunoprecipitation in vitro kinase assay. B, PhosphorImager analysis of phosphorylated MEK-1. Quantitation of the [32P]MEK band indicated 4-4.5- and 5-6-fold increases in MEK phosphorylation activity in Raf immunoprecipitates from carbachol- and EGF-stimulated cells, respectively. Five independent experiments gave similar results.
Basal incubated with 30 pCi/ml S2PI for 6 h and then stimulated with either carbachol or EGF (30 ng/ml) were used for the indicated times and treatment with pertussis toxin (PTx). Pertussis toxin, in two independent experiments, completely inhibited carbachol but not EGF stimulation of Ras activation.

DISCUSSION

It has been generally assumed that heterotrimeric G proteins coupled to seven-membrane-spanning receptors utilized signal transduction pathways that were largely independent of Ras. This assumption was based primarily on the known Ras-independent effectors regulated by heterotrimeric G proteins such as adenylylcyclase, phospholipase Cß, and K⁺ channels (4, 5, 9, 10). There is a growing awareness, however, that G protein-coupled signal transduction pathways can exert a strong growth regulatory control in selected cell types (12). This is particularly true for the G protein-coupled thrombin and LPA receptor control of mitogenesis in fibroblasts (7, 8). Both the thrombin- and LPA receptor-mediated responses seem to involve more than one G protein. For example, thrombin receptor responses have been elegantly shown to involve both G₁ and G₃ (28, 29). The LPA receptor appears to be similarly coupled to both G₁ and G₃ (8). The acetylcholine muscarinic m2 receptor (m2R) is significantly more selective than either the thrombin or LPA receptors and couples primarily to G₁ (1). The m2R did not activate phospholipase C activity in the Rat 1a cells used in this study. For this reason we used the m2R to selectively study the ability of G₁-regulated signal transduction pathways to regulate the MAPK regulatory network. Our results unequivocally demonstrate that the m2R receptor, in a G₁-dependent manner, is capable of mediating Ras and Raf activation in Rat 1a cells. Interestingly, expression of the acetylcholine muscarinic m1 receptor, which selectively couples to G₃ and activates phospholipase Cβ (30), in Rat 1a cells did not activate Ras or Raf (not shown).

The linkage between G₁, Ras, and Raf is presently unclear. The most obvious candidate would be a tyrosine kinase activated by G₁. In fact, this has been proposed recently for the LPA and thrombin receptor activation of Ras in Rat 1 and CCL39 cells (31). This response was inhibited 50–75% by 50 μM genistein, a tyrosine kinase inhibitor, inhibited LPA-stimulated DNA synthesis and only partially inhibited MAPK activation (32). Our studies in Rat 1a cells indicate that the m2R activation of Ras, Raf, and MAPK are genistein-insensitive. The basis for the difference between receptors and cell types in sensitivity to tyrosine kinase inhibitor influences on G₁-coupled activation of Ras is not apparent.

The ability of G₁-coupled seven-membrane-spanning recep-
tors to regulate Ras and Raf in different cell types is presently unclear. Based on the multiple proteins required for tyrosine kinase receptor activation of Ras (33, 34), however, it is probable that this response will not be observed in all cell types. This would predict a unique effector for Gi expressed in some, but not all, cell types capable of integrating Gi with the Ras activation pathway. The contribution of Ras and Raf regulation by Gi will have to be considered when defining the physiological responses of Gi-coupled seven-membrane-spanning receptors.

REFERENCES

1. Parker, E. M., Kameyama, K., Higashijima, T., and Ross, E. M. (1991) J. Biol. Chem. 266, 519–527
2. Hescheler, J., Rosenthal, W., Trautwein, W., and Schultz, G. (1987) Nature 325, 445–447
3. Homburger, V., Brabet, P., Audigier, Y., Pantaloni, C., Bockaert, J., and Rout, B. (1987) Mol. Pharmacol. 31, 313–319
4. Gilman, A. G. (1987) Annu. Rev. Biochem. 56, 615–649
5. Yatani, A., Mattera, R., Codina, J., Graf, R., Okabe, K., Padrell, E., Iyengar, R., Brown, A. M., and Birnbaumer, L. (1988) Nature 336, 680–682
6. Sarnes, M., and Mostov, K. (1992) Mol. Biol. Cell 3, 1317–1328
7. Seuwens, K., Kahan, C., Hartmann, T., and Pouysegu J. (1990) J. Biol. Chem. 265, 22292–22299
8. van Corven, E. J., Grokken, A., Jalink, K., Eicholtz, T., and Moolenaar, W. H. (1998) Cell 90, 45–54
9. Wang, W.-J., and Gilman, A. G. (1992) Cell 70, 869–872
10. Camps, M., Carozzi, A., Schnabel, P., Scheer, A., Parker, P. J., and Gierschik, P. (1992) Nature 360, 684–689
11. Gupta, S. K., Gallego, C., Lowndes, J. M., Pleiman, C. P., Sable, C., Esfeder, B. J., and Johnson, G. L. (1992) Mol. Cell. Biol. 12, 190–197
12. Gupta, S. K., Gallego, C., and Johnson, G. L. (1992) Mol. Cell. Biol. 3, 123–128
13. Wood, R. W., Sarneski, C., Roberts, T. M., and Blenis, L. (1992) Cell 68, 1040–1050
14. Lin, L.-L., Wartmann, M., Lin, A. Y., Kopf, J. L., Seth, A., and Davis, R. J. (1993) Cell 72, 269–278
15. Nemenoff, R. A., Winitz, S., Qian, N. X., Van Patten, V., Johnson, G. L., and Heasley, L. E. (1993) J. Biol. Chem. 268, 1960–1964
16. Seth, A., Alvarez, E., Gupta, S., and Davis, R. J. (1991) J. Biol. Chem. 266, 23521–23524
17. Alvarez, E., Northwood, I. C., Gonzalez, P. A., Latour, D. A., Seth, A., Abate, C., Currim, T., and Davis, R. J. (1991) J. Biol. Chem. 266, 15277–15285
18. Cobb, M. H., Boulton, T. G., and Robbins, D. J. (1991) Cell Regul. 2, 965–978
19. Gupta, S. K., Gallego, C., Johnson, G. L., and Heasley, L. E. (1992) J. Biol. Chem. 267, 7987–7990
20. Gardner, A. M., Vaillancourt, R. R., and Johnson, G. L. (1993) J. Biol. Chem. 268, 1796–1799
21. Sato, T., Endo, M., Nakafuku, M., Nakamura, S., and Kaziyo, Y. (1990) Proc. Natl. Acad. Sci. USA 87, 5963–5967
22. Crews, C. M., Alessandri, A., and Eriksson, R. L. (1992) Science 258, 478–480
23. Kyriakis, J. M., App, H., Zhang, X.-F., Banerjee, P., Brautigam, D. L., Rapp, U. R., and Avruch, J. (1992) Nature 358, 417–421
24. Chambrard, J. G., Paris, S., L’Alleman, G., and Pouysegu, J. (1987) Nature 326, 800–803
25. Moodie, T. A., Willumsen, B. M., Weber, M. J., and Wolfman, A. (1993) Science 260, 1658–1661
26. Kolch, W., Heidecker, G., Lloyd, P., and Rapp, U. R. (1991) Nature 349, 426–428
27. Akaiwama, T., Ishida, J., Nakagawa, S., Ogawa, H., Watanabe, S., Itoh, N., Shibuya, M., and Fukushima, K. (1987) J. Biol. Chem. 262, 5592–5595
28. Hung, D. T., Wong, Y. H., Yu, T.-K. H., and Coughlin, S. R. (1992) J. Biol. Chem. 267, 29631–29634
29. La Morte, V. J., Harootunian, A. T., Spiegel, A. M., Tisen, R. Y., and Fermamico, J. R. (1993) J. Cell Biol. 121, 91–99
30. Berstein, G., Blank, J. L., Smrcka, A. V., Higashijima, T., Sternweis, P. C., Exton, J. H., and Ross, E. M. (1992) J. Biol. Chem. 267, 8081–8088
31. van Corven, E. J., Hordijk, P. L., Medema, R. H., Bos, J. L., and Moelenaar, W. H. (1993) Proc. Natl. Acad. Sci. USA 90, 1257–1261
32. Cook, S. J., Rubinfeld, B., Albert, I., and McCormick, F. (1993) EMBO J., in press
33. Egan, S. E., Giddings, B. W., Brooks, M. W., Buday, L., Szilend, M., and Weinberg, R. A. (1993) Nature 363, 45–51
34. Buday, L., and Downward, J. (1993) Cell 73, 611–620

**Fig. 5. Effect of genistein on carbachol and EGF stimulation of MAPK, Raf and Ras.** Rat 1a cells serum-starved overnight were incubated with 300 μM genistein for 30 min prior to challenge with 100 μM carbachol, 30 ng/ml EGF, or buffer alone (Basal). Cells were challenged for 3 min with carbachol for MAPK and Raf assays and 1 min for measurement of Ras activation. Cells were treated with EGF for 3 min for each assay. Treatment of Rat 1a cells with 100 μM genistein for 30 min had no effect on either EGF or carbachol stimulation of MAPK, Raf, or Ras (not shown), whereas 300 μM genistein inhibited EGF but not carbachol stimulation of MAPK, Raf, and Ras. A, MAPK activity measurements are representative of two independent experiments. B, PhosphorImager quantitation of Raf activity represents the results from three independent experiments (mean ± S.D.). Each of the three experiments, the results were normalized to either basal or basal + genistein treatment. C, PhosphorImager quantitation of Raf activity averaged from three independent experiments (mean ± S.D.). D, phosphotyrosine immunoblot of MAPK from carbachol- and EGF-stimulated cells in the presence or absence of genistein.