Proc. Natl. Acad. Sci. USA
Vol. 88, pp. 4703–4707, June 1991
Cell Biology

Muscarinic acetylcholine receptor subtypes as agonist-dependent oncogenes

(malignant/transformation/second messengers/G proteins/neurotransmitter)

J. SILVIO GUTKIND*†, ELIZABETH A. NOVOTNY‡, MARK R. BRANN‡, AND KEITH C. ROBBINS*

*Laboratory of Cellular Development and Oncology, National Institute of Dental Research; and †Laboratory of Molecular Biology, National Institute of Neurological Disorders and Stroke, National Institutes of Health, Bethesda, MD 20892

Communicated by Bernhard Witkop, March 11, 1991 (received for review January 10, 1991)

ABSTRACT **We have evaluated the muscarinic acetylcholine family of G protein-coupled receptors (mAChRs) for their oncogenic potential. These receptors are preferentially expressed in postmitotic cells, transducing signals specified by their endogenous agonist, the neurotransmitter acetylcholine. Cells transfected with individual human mAChR genes were morphologically indistinguishable from parental NIH 3T3 cells in the absence of agonist. In contrast, when cultures were supplemented with carbachol, a stable analog of acetylcholine, foci of transformation readily appeared in m1, m3, or m5 but not in m2 or m4 mAChRs transfectants. Receptor expression was verified by ligand binding and was similar for each transfected culture. Transformation was dose-dependent and required only low levels of receptor expression. In transformation-competent cells, agonist induced phosphatidylinositol hydrolysis, whereas in m2 or m4 transfectants, receptors were coupled to the inhibition of adenylyl cyclase. These findings demonstrate that mAChRs linked to phosphatidylinositol hydrolysis can act as conditional oncogenes when expressed in cells capable of proliferation.**

Receptors for polypeptides, such as epidermal growth factor and platelet-derived growth factor, can induce cellular transformation when constitutively activated (1–4). Structural mutations or unregulated availability of ligand are mechanisms known to account for their transforming activity. These receptors are prototypes of a class that mediate signal transduction by virtue of an intrinsic protein-tyrosine kinase activity (2, 5, 6). When the *mas* oncogene was discovered, a class of cell-surface receptors lacking protein-tyrosine kinase domains was also implicated in cellular transformation. The *mas* oncogene product has a structural motif characteristic of receptors that mediate signal transduction by coupling to GTP-binding proteins (G proteins) (7). Although *mas* has a weak focus-inducing activity *in vitro*, cells transfected with this gene are highly tumorigenic in nude mice (7). More recently, G protein-coupled serotonin receptors have been shown to convert fibroblasts to a tumorigenic state (8). Because in these latter cases exogenous ligand is not required for transformation, either these genes encode aberrant receptors or endogenous ligands are responsible for their activation. Thus, ligand independence has limited the study of the mechanism by which these receptors mediate transformation.

In the present study, we have directly tested the hypothesis that normal G-protein-coupled receptors can induce agonist-dependent neoplastic transformation. We chose a family of cell-surface neurotransmitter receptors, human muscarinic acetylcholine receptors (mAChRs), which possess sequence homology with both *mas* and the serotonin receptor (9–12). Muscarinic receptors are preferentially expressed in neurons and other postmitotic cells, and they transduce signals specified by their endogenous agonist, the neurotransmitter acetylcholine. The mAChR family consists of five distinct but highly homologous subtypes (m1–m5), which are encoded by five separate genes (9–12). Individual mAChRs have functional differences when expressed by cultured cells. Odd-numbered mAChRs potently stimulate phosphatidylinositol (PtdIns) metabolism, arachidonic acid release, and open Ca^{2+}-dependent potassium channels by coupling with a pertussis toxin insensitive G protein. m2 and m4 mAChRs couple to a pertussis toxin-sensitive G protein to inhibit adenylyl cyclase (13–15). Depending upon assay conditions, m1, m3, and m5 mAChRs have been linked to both increases (16) and decreases (17) in mitogenesis.

MATERIALS AND METHODS

Transfection. Plasmid DNA transfection of NIH 3T3 (18) cells was performed by the calcium phosphate precipitation technique, as modified by Wigler *et al.* (19). Mass populations expressing the transfected gene were selected for ability to grow in the presence of Geneticin (G418) (GIBCO); transformed foci were scored after 2–3 weeks. Individual G418 colonies or transformed foci were isolated with the aid of cloning cylinders and maintained in Dulbecco's modified Eagle medium/10% calf serum.

DNA Constructs. Human mAChRs genes (9–12) were inserted into an expression vector, pDS, which contained a dominant selectable marker, *neo* (20). pDS v-*fgr* was derived from pSV2v-*fgr*, which contained the biologically active Gardner-Rasheed feline sarcoma virus proviral genome (21, 22).

Analysis of Receptor Expression. Cell membranes were isolated, and binding was assayed as described (23). Saturation experiments were done at 10 different concentrations of L-[*N-methyl*-^{3}H]scopolamine methyl chloride ([^{3}H]NMS; 1 pM–1 nM) in duplicate. Data were fit by using nonlinear regression with the equation $y = B_{max}X^N/K_d/(1 - B_{max}X^N/K_d)$ with the program KAL on a Mac II computer, where y is specific binding and X is free ligand concentration. Nonspecific binding was determined with 10 μM atropine.

PtdIns Hydrolysis. Subconfluent NIH 3T3 transfectants were incubated in 24-well plates with *myo*-[^{3}H]inositol at 1 μCi/ml (1 Ci = 37 GBq) for 48 hr. Immediately before an experiment, cells were washed twice with Eagle's medium/10 mM LiCl and incubated for 10 min at room temperature. Cells were treated for 1 hr with 0.5 ml of medium/10 mM LiCl containing experimental agents. Inositol phosphates were extracted with 0.5 ml of ice-cold 10% (wt/vol) trichloroacetic acid and analyzed by ion-exchange chroma-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: PtdIns, phosphatidylinositol; [^{3}H]NMS, L-[*N-methyl*-^{3}H]scopolamine methyl chloride; mAChR, muscarinic acetylcholine receptor.

†To whom reprint requests should be addressed.

tography by the method of Berridge *et al.* (24). Ten concentrations of carbachol (50 nM–333 μM) were used to determine ED_{50} and maximal responses.

cAMP Assays. Transfected NIH 3T3 cells were grown to 90% confluence in 24-well plates. Medium was replaced with 0.25 ml of Eagle's medium/1 mM 3-isobutyl-1-methylxanthine containing experimental agents. The reaction was stopped after 10 min of incubation at room temperature by adding 0.25 ml of an ice-cold solution containing 0.1 M HCl and 1 mM $CaCl_2$. cAMP levels were determined by using a Gammaflow automated RIA for acetylated cAMP (Atto Instruments, Potomac, MD).

RESULTS

Conditional Transformation of Certain mAChR Transfectants. Expression plasmids carrying each of the human mAChRs were transfected into NIH 3T3 cells, a murine fibroblast lacking endogenous mAChRs (18). Expression of each mAChR gene was driven by the Moloney leukemia virus long terminal repeat, a potent transcriptional promoter in NIH 3T3 cells. The same constructs also contained a dominant selectable marker, *neo* (20), that conferred resistance to Geneticin (G418), thereby permitting determination of transfection efficiencies. As shown in Table 1, the number of G418-resistant colonies was nearly identical for all plasmid DNAs tested, except for m5, which was not further examined. In contrast, transforming activity varied widely among DNA constructs. The v-*fgr* oncogene (21, 22) efficiently induced focus formation, whereas mAChR transfectants appeared morphologically indistinguishable from parental NIH 3T3 or vector-transfected cells (Table 1). However, when maintained in the presence of carbachol, a stable analog of acetylcholine, foci of transformation readily appeared in cultures transfected with m1 or m3 but not with m2 or m4 mAChR genes (Table 1). Focus formation increased as a function of agonist concentration (Table 1), and carbachol-induced transformation was prevented by the muscarinic antagonist atropine (Fig. 1).

Agonist Requirement for Maintenance of the Transformed State. To determine whether transformation of m1 and m3 AChR transfectants required agonist for maintenance of their transformed state, foci were picked and replated on a lawn of untransfected NIH 3T3 cells in medium lacking carbachol.

Table 1. Focus-forming activity of cloned human muscarinic acetylcholine receptors

DNA clone	Focus-forming activity in carbachol per pmol of DNA			Colony-forming activity per pmol of DNA
	0 M	1 μM	100 μM	
pDS	<1	<1	<1	2800
pDS m1	<1	36	580	2400
pDS m2	<1	<1	<1	2400
pDS m3	<1	34	280	1900
pDS m4	<1	<1	<1	2600
pDS m5	<1	<5	47	600
pDS v-*fgr*	1900	–	–	3700

Human mAChRs genes or v-*fgr* were inserted into an expression vector, pDS, and 0.05–1 μg of plasmid DNA was transfected into NIH 3T3 murine fibroblasts (18) as described (19). Media containing various concentrations of the muscarinic cholinergic agonist, carbachol, or the muscarinic cholinergic antagonist, atropine (10 μM), were replaced every 2 days. Foci of transformation were scored after 2–3 weeks. Efficiency of transfection was determined by scoring *neo*-resistant colonies in medium containing G418 (0.750 mg/ml). No foci of transformation were observed in cells maintained with atropine, and atropine abolished carbachol-induced focus-formation (data not shown). Results represent the averages of three independent experiments.

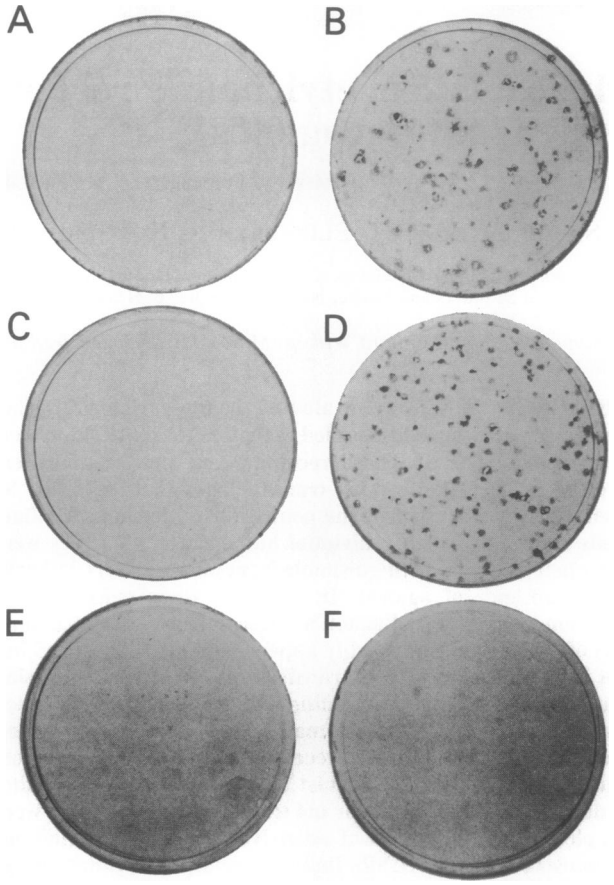

FIG. 1. Focus assay after transfection of NIH 3T3 cells with mAChR DNAs. NIH 3T3 cells were transfected with 1 μg of pDS (*A*), 0.3 μg of pDS v-*fgr* (*B*), or 1 μg of pDS m1 (*C–F*) plasmid DNA. Cultures were maintained in Dulbecco's modified Eagle's medium (*A–C*), supplemented with 100 μM carbachol (*D*), 10 μM atropine (*E*), or 100 μM carbachol and 10 μM atropine in combination (*F*). Plates were stained 3 weeks after transfection.

Fig. 2 shows that under these conditions, cells returned to their nontransformed morphology. In contrast, foci arose when the culture medium was supplemented with carbachol. Furthermore, focus-derived cells could form large colonies in soft agar in the presence, but not in the absence, of agonist (data not shown). Thus, m1 and m3 mAChR transfectants possessed properties of fully malignant cells. These findings showed that m1 and m3 mAChRs were potent transforming agents and that agonist was required for induction and maintenance of malignant transformation.

Verification of mAChR Expression in NIH 3T3 Transfectants. To directly examine mAChR expression in transfected cells, several m1 and m3 foci were cloned and evaluated for receptor expression by binding of a labeled nonselective mAChR antagonist, [^{3}H]NMS. Receptor numbers were found to be higher in cloned m1 and m3 foci as compared with parental Geneticin-selected mass cultures (Table 2). This finding suggested that high levels of receptor expression were required for transforming activity. Binding to m2- and m4-transfected mass cultures was similar to that for m3 transfectants (Table 2). Thus, the lack of foci in m2- and m4-transfected cultures demonstrated an intrinsic difference between m1 and m3 as compared with m2 and m4 mAChRs. We also investigated binding characteristics of mAChRs expressed in NIH 3T3 cells. Dissociation constants and total binding sites for representative G418-selected clones revealed K_d values of 63, 82, 38, and 26 pM for m1–m4 receptors, respectively. These affinities are nearly identical to those described in other cell lines (16, 17, 23).

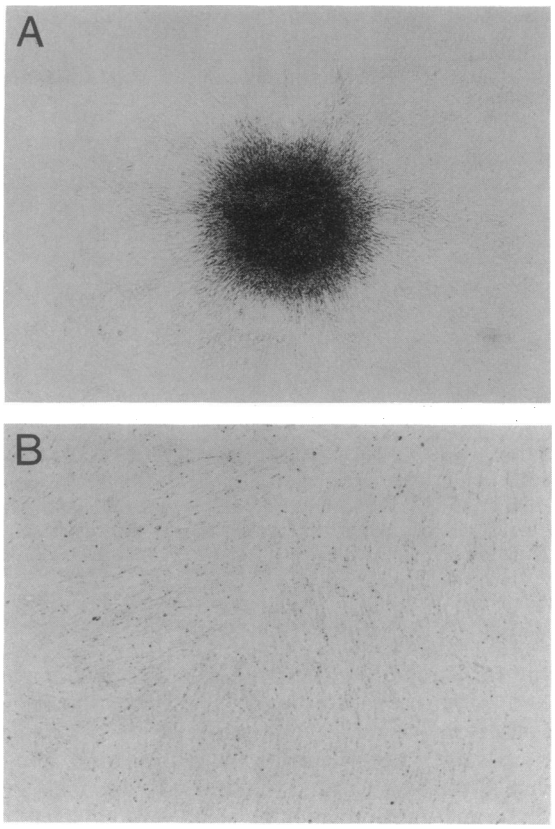

FIG. 2. Reversibility of transformation in NIH 3T3 expressing m1 mAChR. (*A*) Focus of transformation that arose 2 weeks after transfection with 1 μg of pDS m1 DNA in the presence of the muscarinic cholinergic agonist carbachol. (*B*) Focus was isolated with the aid of a cloning cylinder, trypsinized, and plated on a lawn of NIH 3T3 cells in medium lacking carbachol.

Quantitation of Transformation Induced by Carbachol. To explore quantitative aspects of transformation induced by activation of m1 and m3 mAChRs, we devised an assay for ligand-dependent transformation that involved plating mAChR transfectants on a lawn of NIH 3T3 cells. Without carbachol, no foci of transformation were seen. However, when concentrations of the agonist from 100 nM to 1 mM were added to the culture medium, foci arose in <1 week in a clonal m1 transfectant (Fig. 3). Furthermore, the number of foci seen was directly proportional to ligand concentration (Fig. 3, and see below). At a carbachol concentration of 1 mM, 100% of the m1 transfectants plated gave rise to foci of transformation. Under these conditions as well, focus formation was prevented by atropine (10 μM) (data not shown). Taken together, our findings demonstrate that receptor number as well as agonist concentration are direct determinants of transformation in this system.

Table 2. Ligand binding to transfected cells

DNA clone	Binding of [^{3}H]NMS, fmol of [^{3}H]NMS per mg of protein	
	Mass culture	Cloned focus
pDS	<1	
pDS m1	430	640 ± 58*
pDS m2	90	
pDS m3	63	496 ± 146*
pDS m4	40	

Receptor density of each G418-selected mass culture or individual-cloned focus was measured by specific binding of the nonselective mAChR antagonist [^{3}H]NMS to membranes prepared from specified cell lines, as described (13). Nonspecific binding was determined with 10 μM atropine.
*Data are means ± SEM of six cloned foci.

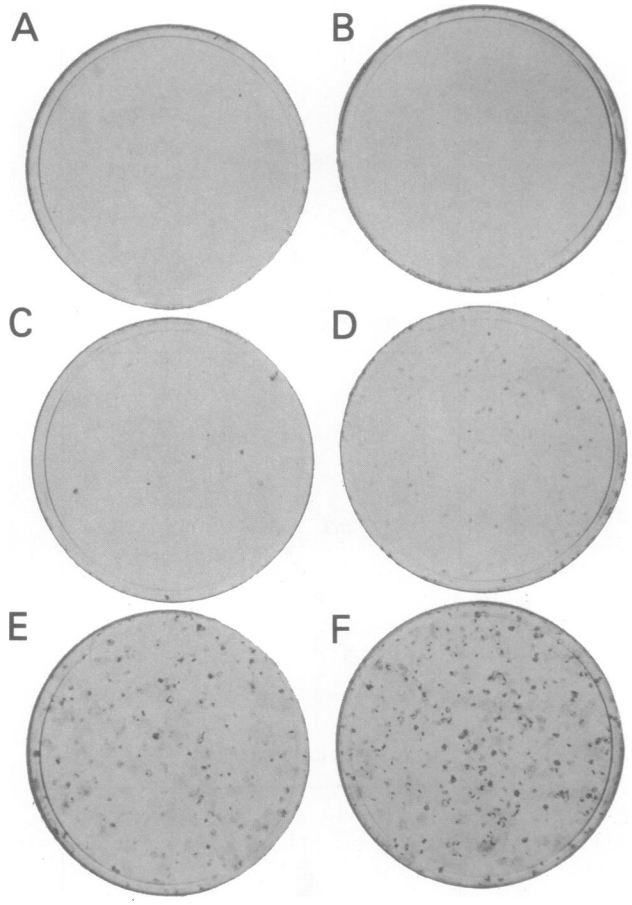

FIG. 3. Ligand-dependent transformation of NIH 3T3 cells expressing m1 or m3 mAChRs. Approximately 200 cells, derived from a clone of G418-selected pS m1-transfected cells expressing 1100 fmol of [^{3}H]NMS-binding sites per mg of protein, were plated together with 2×10^5 untransfected NIH 3T3 cells. Cultures were grown without carbachol (*A*) or with 0.1 μM (*B*), 1.0 μM (*C*), 3.0 μM (*D*), 10 μM (*E*), or 100 μM (*F*) carbachol and stained after 2 weeks.

Second-Messenger Coupling in mAChR Transfectants. To determine whether mAChRs expressed in NIH 3T3 cells were coupled to second-messenger generation systems, transfectants were examined for known biochemical effects in response to agonist. Significant PtdIns hydrolysis was seen in cells expressing m1 or m3 receptors but not in m2 and m4 expressors, even at very high agonist concentration (Fig. 4). Carbachol decreased cAMP levels only in forskolin-treated cells expressing m2 and m4 mAChRs (Fig. 4). Similar results were obtained by using a number of other clones expressing m2 or m4 mAChRs subtypes (data not shown). These findings demonstrate that known biochemical responses characteristic of mAChRs are reflected in our NIH 3T3 cell transfectants. We conclude that only those mAChRs coupling to PtdIns hydrolysis are transforming in NIH 3T3 cells, whereas adenylyl cyclase inhibition is neither necessary nor sufficient to induce focus formation in mAChR transfectants.

Quantitative Relationship Between Focus Formation and Extent of PtdIns Hydrolysis. Observations that transformation as well as PtdIns hydrolysis were dose dependent for m1 and m3 transfectants provided an opportunity to characterize the relationship between the biological and biochemical responses to agonist. Thus, we compared G418-selected NIH 3T3 cell clones expressing comparable levels of each mAChR

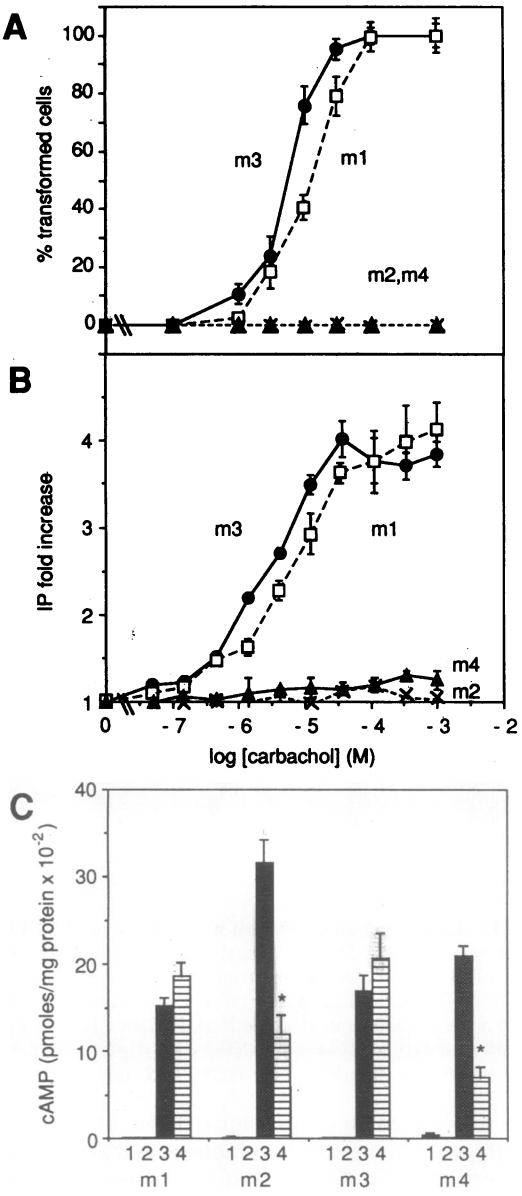

FIG. 4. Effect of carbachol on cell transformation, PtdIns (PI) hydrolysis, and cAMP levels in G418-selected NIH 3T3 clones expressing different mAChR subtypes. Carbachol-induced cell transformation (*A*), activation of PtdCho hydrolysis (*B*), and inhibition of forskolin-stimulated cAMP accumulation (*C*) were measured in cells expressing m1, m2, m3, or m4 mAChRs, as indicated. Receptor expression was 600, 300, 370, and 650 fmol of [^{3}H]NMS binding sites per mg of protein for m1–m4 mAChRs, respectively. Cell transformation was determined by plating cells on a lawn of nontransformed NIH 3T3 cells. Cells were maintained with the indicated concentrations of carbachol, and foci were counted 2 weeks later. Each data point represents the average ± SEM of triplicate plates from two to three separate experiments, expressed as the ratio of foci to the number of plated cells × 100. PtdIns hydrolysis was determined as described. Each data point represents the average ± SEM for triplicate samples from three to four separate experiments, expressed as ratio of ^{3}H-labeled PtdIns accumulated in stimulated versus unstimulated cells. Atropine (10 μM) prevented the carbachol-dependent accumulation of ^{3}H-labeled PtdIns (data not shown). Inhibition of adenylyl cyclase was determined by measuring the effect of carbachol on cAMP accumulation induced by forskolin, in the presence of isobutylmethylxanthine (100 μM). Cells, treated for 10 min with saline solution (bar 1) or 1 mM carbachol (bar 2), did not accumulate detectable cAMP. Treatment with 10 mM forskolin alone (bar 3) or in combination with 1 mM carbachol (bar 4) is also shown. cAMP was measured by RIA as described. *, $P < 0.01$ versus forskolin alone. Data are means ± SEM of three replicate determinations.

Table 3. Effect of carbachol on PtdIns hydrolysis and transformation

Gene	Binding, fmol [^{3}H]NMS*	PtdIns hydrolysis: Maximal activation, fold	PtdIns hydrolysis: EC_{50}, μM	Transformation: Maximal transformation, %	Transformation: EC_{50}, μM
neo	0	1.0	ND	0	ND
m1	38	1.3	1	2	†
	612	2.2	1	>95	10
	919	6.5	0.5	>95	6
m2	308	1.2	†	0	ND
m3	60	1.6	3	3	†
	373	2.1	1	>95	3
	1309	4.1	3	>95	8
m4	654	1.3	40	0	ND

G418-selected clones expressing different mAChR subtypes were tested for PtdIns hydrolysis and cell transformation in response to carbachol at 50 nM–333 μM. Results represent the averages from three to four experiments. SE was <5% of the mean for each determination. EC_{50}, half-maximal concentration. ND, not determined.

*Binding is indicated in fmol of [^{3}H]NMS per mg of protein.

†Values were too low to be determined with sufficient accuracy.

subtype for PtdIns hydrolysis and focus formation in response to various concentrations of agonist. As shown in Fig. 4, we observed that curves for PtdIns hydrolysis were nearly superimposable upon those for focus formation. We then determined the half-maximal dose (EC_{50}) for both PtdIns hydrolysis and transformation by using several clonal transfectants selected for variations in receptor expression. For each clone examined, the half-maximal concentration of carbachol required for cell transformation was nearly identical to that necessary to stimulate PtdIns hydrolysis (Table 3). Furthermore, the maximal PtdIns hydrolysis response was proportional to the number of mAChRs expressed by respective m1 or m3 clonal transfectants.

Very few foci were seen in cells expressing low numbers of m1 or m3 mAChR. In contrast, >95% of m1 or m3 transfectants, binding >612 or 373 fmol of [^{3}H]NMS per mg of cellular protein, respectively, gave rise to foci of transformation. A similar analysis of the remaining m1- or m3-transfected clones revealed that below a level of 90 to 100 fmol of [^{3}H]NMS binding per mg of protein, no effective agonist-induced focus formation was observed. These findings suggest a low threshold of receptor expression for agonist-induced transformation. Taken together, our findings also demonstrate a close relationship between the activation of PtdIns hydrolysis and cellular transformation.

DISCUSSION

The protein products of the *mas* oncogene and 5-hydroxytryptophan 1c (serotonin) receptors are transforming in NIH 3T3 cells and are thought to be coupled to PtdIns hydrolysis (7, 8). However, correlation between this biochemical response and transformation has not been directly demonstrated. Furthermore, overexpression of G protein-coupled receptors can overcome the specificity of coupling to second messengers (25, 26). Thus, involvement of particular biochemical pathways in cellular transformation induced by G protein-coupled receptors cannot be inferred from biochemical responses measured in other systems. In the present study, we have demonstrated a close relationship between agonist-induced transformation and extent of PtdIns turnover. Our findings suggest that other G protein-coupled receptors linked to PtdIns hydrolysis might also possess transforming potential.

Inhibition of adenylyl cyclase has been implicated in the mitogenic response to substances, such as serotonin or lysophosphatidic acid (27–29), and thus also to transformation. In the present study, we have shown that receptors actively inhibiting adenylyl cyclase do not induce focus formation or anchorage-independent growth, whereas stimulation of receptors coupled to PtdIns hydrolysis are highly transforming. We have also shown that the cAMP response to forskolin was not decreased in cells expressing m1 or m3 mAChRs. Thus, in contrast to previous suggestions (27–29), our present findings establish that inhibition of adenylate cyclase is neither sufficient nor necessary for transformation of murine fibroblasts.

We cannot rule out the possibility that second messengers other than PtdIns hydrolysis may be involved in the transformation response seen. Activation of mAChRs may transform NIH 3T3 cells by coupling to arachidonic acid release and prostaglandin synthesis or by altering the behavior of ion channels (13–15, 17). Alternatively, the *ras* oncogene product (a GTP-binding protein) has been suggested to alter G protein-coupled receptor functioning (30–32), including m2 mAChR-mediated activation of cardiac K^+ channels (33). If so, a provocative linkage may exist between G protein-coupled receptors and p21*ras*. Finally, tyrosine phosphorylation, one of the hallmarks of oncogene-induced transformation, can be induced in neutrophils or platelets by chemotactic agents (34) or thrombin (35, 36), respectively, both of which can activate G protein-coupled receptors (37–39). Thus, further investigation will be required to determine the contributions of each of these various biochemical processes to malignant transformation.

Expression of mAChRs as well as other G protein-coupled receptors is limited to neurons or other terminally differentiated cells. In general, this family of receptors transduces signals involved in differentiated functions performed by mature cells. Thus, the transforming action of these receptors, when activated in fibroblasts, suggests that biochemical responses normally signaling differentiated functions might contribute to naturally occurring tumorigenesis when elicited in immature cells having proliferative capacity.

1. DiFiore, P. P., Pierce, J. H., Fleming, T. P., Hafan, R., Ullrich, A., King, C. R., Schlessinger, J. & Aaronson, S. A. (1987) *Cell* **51**, 1063–1070.
2. Downward, J., Yarden, Y., Mayes, E., Scrace, G., Totty, N., Stockwell, P., Ullrich, A., Schlessinger, J. & Waterfield, M. D. (1984) *Nature (London)* **307**, 521–527.
3. Leal, F., Williams, L. T., Robbins, K. C. & Aaronson, S. A. (1985) *Science* **230**, 327–330.
4. Velu, T. J., Beguinot, L., Vass, W. C., Willingham, M. C., Merlino, G. T., Pastan, I. & Lowy, D. R. (1987) *Science* **238**, 1408–1410.
5. Yarden, Y., Escobedo, J. A., Kuang, W. J., Yang-Feng, T. L., Daniel, T. O., Tremble, P. M., Chen, E. Y., Ando, M. E., Harkins, R. N., Francke, U., Fried, V. A., Ullrich, A. & Williams, L. T. (1986) *Nature (London)* **323**, 226–232.
6. Matsui, T., Heidaran, M., Miki, T., Popescu, N., La Rochelle, W., Kraus, M., Pierce, J. & Aaronson, S. (1989) *Science* **243**, 800–804.
7. Young, D., Waitches, G., Birchmeier, C., Fasano, O. & Wigler, M. (1986) *Cell* **45**, 711–719.
8. Julius, D., Livelli, T. J., Jessell, T. M. & Axel, R. (1989) *Science* **244**, 1057–1062.
9. Kubo, T., Fukuda, K., Mikami, A., Maeda, A., Takahashi, H., Mishina, M., Haga, T., Haga, K., Ichiyama, A., Kangawa, K., Kojima, M., Matsuo, H., Hirose, T. & Numa, S. (1986) *Nature (London)* **323**, 411–416.
10. Peralta, E. G., Ashkenazi, A., Winslow, J. W., Smith, D. H., Ramachandran, J. & Capon, D. J. (1987) *EMBO J.* **6**, 3923–3929.
11. Bonner, T. I., Burkley, N. J., Young, A. C. & Brann, M. R. (1987) *Science* **237**, 527–532.
12. Bonner, T. I., Young, A. C., Brann, M. R. & Burkley, N. J. (1988) *Neuron* **1**, 403–410.
13. Jones, S. V. P., Barker, J. L., Burkley, N. J., Bonner, T. I., Collins, R. M. & Brann, M. R. (1988) *Mol. Pharmacol.* **34**, 421–426.
14. Peralta, E. G., Ashkenazi, A., Winslow, J. W., Ramachandran, J. & Capon, D. J. (1988) *Nature (London)* **334**, 434–437.
15. Fukuda, K., Higashida, H., Kubo, T., Maeda, A., Akiba, I., Bujo, H., Mishina, M. & Numa, S. (1988) *Nature (London)* **335**, 355–358.
16. Ashkenazi, A., Ramachandran, J. & Capon, D. J. (1989) *Nature (London)* **340**, 146–150.
17. Conklin, B. R., Brann, M. R., Buckley, N. J., Ma, A. L., Bonner, T. I. & Axelrod, J. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 8698–8702.
18. Jainchill, J. L., Aaronson, S. A. & Todaro, G. J. (1969) *J. Virol.* **4**, 549–553.
19. Wigler, M., Silverstein, S., Lee, L. S., Pellicer, A., Cheng, Y. C. & Axel, R. (1977) *Cell* **11**, 223–232.
20. Korman, A. J., Frantz, J. D., Strominger, J. L. & Mulligan, R. C. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 2150–2154.
21. Naharro, G., Tronick, S. R., Rasheed, S., Gardner, M. B., Aaronson, S. A. & Robbins, K. C. (1983) *J. Virol.* **47**, 611–619.
22. Naharro, G., Robbins, K. C. & Reddy, E. P. (1984) *Science* **223**, 63–66.
23. Buckley, N. J., Bonner, T. I., Buckley, C. M. & Brann, M. R. (1989) *Mol. Pharmacol.* **35**, 469–476.
24. Berridge, M. J., Dawson, M. C., Downes, C. P., Heslop, J. P. & Irvine, R. F. (1983) *Biochem. J.* **212**, 473–482.
25. Ashkenazi, M., Winslow, J. W., Peralta, E. G., Peterson, G. L., Schimerlik, M. I., Capon, D. J. & Ramachandran, J. (1987) *Science* **238**, 672–675.
26. Stein, R., Pinkas-Kramarski, R. & Sokolovsky, M. (1988) *EMBO J.* **7**, 3031–3035.
27. Seuwen, K., Magnaldo, I. & Pouyssegur, J. (1988) *Nature (London)* **335**, 254–256.
28. Seuwen, K. & Pouyssegur, J. (1990) *Biochem. Pharmacol.* **39**, 985–990.
29. van Corven, E. J., Groenink, A., Jalink, K., Eichholtz, T. & Moolenaar, W. H. (1989) *Cell* **59**, 45–54.
30. Wakelam, M. J., Davies, S. A., Houslay, M. D. & McKay, I. (1986) *Nature (London)* **323**, 173–176.
31. Parries, G., Hoebel, R. & Racker, E. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 2648–2652.
32. Alonso, T., Srivastava, S. & Santos, E. (1990) *Mol. Cell. Biol.* **10**, 3117–3124.
33. Yatani, A., Okabe, K., Polakis, P. & Halenbeck, R. (1990) *Cell* **61**, 769–776.
34. Huang, C.-K., Laramee, G. L. & Casnellie, J. E. (1988) *Biochem. Biophys. Res. Commun.* **151**, 794–801.
35. Ferrel, J. E., Jr., & Martin, G. S. (1988) *Mol. Cell. Biol.* **8**, 3603–3610.
36. Golden, A. & Brugge, J. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 901–905.
37. Bokoch, G. M. & Gilman, A. G. (1984) *Cell* **39**, 301–308.
38. Koo, C., Lefkowitz, R. J. & Snyderman, R. (1983) *J. Clin. Invest.* **72**, 748–753.
39. Brass, L. F., Laposata, M., Banga, H. S. & Rittenhouse, S. E. (1986) *J. Biol. Chem.* **261**, 16838–16847.