Identification and Molecular Characterization of a m5 Muscarinic Receptor in A2058 Human Melanoma Cells

COUPLING TO INHIBITION OF ADENYLYL CYCLASE AND STIMULATION OF PHOSPHOLIPASE A2*

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We report the identification and biochemical characterization of an endogenous m5 muscarinic acetylcholine receptor (mAChR) in the A2058 human melanoma cell line. This is the first demonstration of a mAChR outside the central nervous system. The unusual effector coupling of this endogenous mAChR is presented. The coding region amplified by polymerase chain reaction was identical to the known mAChR sequence. Binding studies indicated a Kd of 99 ± 6 pm and a Bmax of 45 ± 4 fmol/mg membrane protein. This mAChR coupled to stimulation of arachidonic acid release and to a 50% inhibition of forskolin-stimulated cAMP accumulation. The inhibition of cAMP production was insensitive to pertussis toxin treatment, but was dependent upon extracellular calcium. In contrast to the odd mAChR pattern, no cAMP was produced in response to carbachol (CC) stimulation. Moreover, no release of inositol phosphates could be measured after CC treatment despite the presence of at least 2 phospholipase C isoforms in A2058 cells. CC-stimulated arachidonic acid release (EC50 = 17.8 ± 0.1 μM) was dependent upon external Ca2+, with marked reduction after coinubcation with EGTA, Ca2+, or high doses of verapamil (IC50 = 166 μM) or diltiazem (IC50 = 243 μM). Brief exposure to phorbol 12-myristate 13-acetate augmented CC-stimulated arachidonic acid release, whereas prolonged phorbol 12-myristate 13-acetate treatment resulted in down-regulation of release. Activation of the mAChR resulted in Ca2+ influx that was attenuated by muscarinic antagonism and removal of extracellular Ca2+. A2058 cells exposed to CC had no alteration of cell shape or growth potential in monolayer culture, however, a statistically significant reduction in density-independent growth was observed over the range of CC concentrations from 0.1 to 100 μM. This endogenous mAChR has a novel signal transduction coupling profile and receptor activation reduces clonogenic potential.

The muscarinic acetylcholine receptor (mAChR)3 family consisting of 5 members (m1-m5) is important in the mediation of glandular secretion, pacemaker activity, smooth muscle tone, and neurotransmission (1–6). Its distribution is predominantly in neural tissue or highly innervated smooth muscle-containing tissues. The odd-numbered receptors (m1, m3, and m5) have similar biochemical characteristics consisting of agonist-mediated generation of inositol polyphosphates, release of arachidonic acid, influx and internal release of calcium, tyrosine kinase activation, and production of cAMP (7–15). In contrast, the even-numbered receptors (m2 and m4) are associated with stimulation of cAMP production in response to stimulation of other receptors. m5AChR has been found in brain in low abundance, however, endogenous m5 has only been demonstrated in the brain (1, 2, 4, 19, 20). All other mAChR receptor subtypes are found in the brain as well as other systemic sites. The m2 receptor is abundant in heart muscle and is found in smooth muscles, such as the small and large intestine, trachea, bladder, and uterus (1, 2, 4, 6, 18, 20, 21). In contrast, the odd-numbered receptors are not found in heart, and only the m3 subtype may be found in other smooth muscle-containing organs. Both m1 and m3 are located in glandular tissues such as lacrimal gland, exocrine pancreas, and salivary gland. Functional receptors have been found in a variety of brain cell lines and brain cancer cell lines. An adherent variant small cell lung cancer cell line has been shown to express the m3 subtype receptor. mAChRs have not been reported previously in melanomas, a malignancy of basal layer pigmented neuroepithelial cells.

We now demonstrate and biochemically characterize a m5 AChR in an early passage human melanoma cell line. This m5 receptor is present in extremely low abundance and mediates the inhibition of anchorage independent proliferation. It is functionally associated with a unique combination of signal transduction pathway coupling including calcium mobilization, release of arachidonic acid, inhibition of the generation of cAMP, and lack of inositol polyphosphate production.

EXPERIMENTAL PROCEDURES

Materials—All radioligands for signal transduction and binding studies ([5,6,8,9,11,12,14,15-3H]arachidonic acid and N-(methyl-3H)isocyanate), molecular studies, and the IP3 radioreceptor assay were obtained from Du Pont NEN. Fura-2 AM was from Molecular Probes (Eugene, OR). Reagents for molecular analyses, polymerase chain reaction, cloning, and sequencing were from Life Technologies, Inc./BRL (Gaithersburg, MD), Perkin-Elmer Instruments (Norwalk, CT), Invitrogen Corp. (San Diego, CA), and Amersham Life Sciences. Reagents used in the radioimmunoassay of cAMP were purchased from Dr. Gary
Endogenous Melanoma mAChR with Novel Signal Coupling

Brooker (Department of Biochemistry, Georgetown University School of Medicine, Washington, D.C.). Vitrogen was obtained from Collagen Corp. (Palo Alto, CA). Monodonal anti-bovine phospholipase C (PLC)-I, anti-bovine phospholipase C-1, and anti-phosphotyrosine (4G10) antibodies were obtained from Upstate Biotechnology Inc. (Lake Placid, NY). 125I-Protein A was from Amersham. All other reagents were of reagent-grade.

Cell Culture—The early passage A2058 human melanoma cell line (p<passage 20) was maintained in culture in Dulbecco's modified Eagles medium with 10% fetal calf serum, as described, and used for no more than 8 passages (23). Sublines derived by limiting dilution cloning (A2058–1F5, p<passage 25) were studied preferentially, unless indicated otherwise. This subline has identical growth and functional characteristics as the parental A2058 cell line. Where indicated, CHO cells were used as controls for the muscarinic receptor responses; these cells were maintained in α-minimal essential medium with 10% fetal calf serum.

Molecular Analysis of A2058 Muscarinic Receptor—Polymerase chain reaction, subcloning, and sequencing, followed by Southern blotting or Northern blot analysis, were used to identify the mAChR subtype found in the A2058 cells. Oligonucleotides for PCR were as follows: hu-m5AChR sense primer (bp 340–360: AGCAACGCTTCTGTCATT-GAAC), antisense primer (bp 1555–1435: ATAGCAACAACCATAGCCCA (19)); hu-m2AChR, sense primer (bp 290–307: AGCCTGGCTGTTGTGCAG), antisense primer (bp 1291–1272: GTAGAAGGCCAAGCAAGAAAT), hu-m4AChR, sense primer (bp 554–574: AGCTTTGCGCTTTGGC), antisense primer (bp 1603–1583: GATGAAAGCCAACAGTCACTGACTGAGGTGG). A small m5-specific PCR fragment was isolated from CHO m5 cells for use in Southern blots of the PCR reactions. The primers for this component of the m5AChR were: sense primer (bp 973–993: TCTAGAAGTCCAGGTTAAGGA), antisense primer (bp 1191–1171: ACACGCATTGGTGGCTCCCTG). All PCR fragments were subcloned into TO plasmid for sequencing using the Sequenase kit (Amersham) and subcloning, followed by Southern blot analysis. Total RNA extracted from CHO m1-m5 cells and A2058–1F5 cells were used initially to PCR amplify a segment of the A2058 mAChR. When the initial fragments were sequenced and confirmed the identify of the band in the m5 and 1F5 lanes, Southern analysis was performed using as a probe a 200-bp fragment from the m5AChR coding region. The effect of CC on colony formation in soft agar was determined as described (24, 29). Thirty million A2058 cells were plated into a top 3% agar layer in complete media with or without both agents. After 10 days, colonies were counted under phase-contrast microscopy. Data presented are the mean ± S.E. of three independent experiments. Unpaired Student's t test was used for statistical analysis.

Results

PCR Cloning and Sequence Analysis of mAChR in A2058 Cells

Oligonucleotides from conserved transmembrane domains were used initially to PCR amplify a segment of the A2058 mAChR. When the initial fragments were sequenced and confirmed to have high homology to the m5 subtype, further PCR amplification reactions used more selective oligonucleotides. The effect of CC on colony formation in soft agar was determined as described (24, 29). Thirty million A2058 cells were plated into a top 3% agar layer in complete media with or without both agents. After 10 days, colonies were counted under phase-contrast microscopy. Data presented are the mean ± S.E. of three independent experiments. Unpaired Student's t test was used for statistical analysis.

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show that there is only one species of AChR in the A2058 cells, further PCR amplification was done using oligonucleotides selective to the even-numbered AChRs. A band of approximate size of 900 bp was demonstrated only in the CHO m2 and m4 cells after amplification with m2/m4 oligonucleotide pairs. Southern blot hybridization of those gels with an internal and selective m2/m4 oligonucleotide confirmed that the 900-bp transcript was present only in the CHO m2 and m4 cells and not in the A2058-1F5 cells. The full coding length of the A2058 AChR has been amplified, subcloned, and sequenced and was found to be homologous to that of the hu-m5AChR. These results demonstrate that the AChR on the A2058 cells is genetically a m5 muscarinic receptor.

Receptor Characterization

m5 receptor expression level and receptor affinity were determined by radioligand binding experiments. Saturation binding experiments were performed in which N-[methyl-3H]scopolamine was used to define total binding and unlabeled 10 μM atropine was added to saturate specific binding. Fig. 2 shows the binding characteristics of this receptor. Greater than 90% specific binding was observed in A2058 plasma membranes with binding constants of $K_d = 99 \pm 6 \text{ pM}$ and $B_{max} = 45 \pm 4 \text{ fmol/mg}$ of membrane protein.

Signal Transduction Coupling of Endogenous mACHR in A2058 Cells

Inhibition of forskolin-stimulated cAMP production—Forskolin treatment of A2058 cells resulted in production of cAMP as shown in Fig. 3A. No production of cAMP was observed when A2058-1F5 cells were exposed to CC in concentrations up to 1 μM (data not shown), as would be expected for an odd-numbered muscarinic receptor signaling profile. In contrast, CC exposure inhibited the stimulation of cAMP production in response to forskolin in a dose dependent fashion, but only by 60%. This was investigated further by exposure to pertussis toxin and by removal of extracellular calcium from the reaction medium. When cells were pretreated with pertussis toxin for up to 18 h prior to activation with forskolin, with or without additional CC, no reversal of the effect of CC on cAMP production was demonstrated. Treatment with pertussis toxin did not effect the basal levels of cAMP produced in response to CC treatment alone but increased the forskolin-stimulated cAMP, suggesting the relief of an tonic inhibition by the pertussis toxin incubation. Removal of extracellular calcium from the reaction mixture resulted in abrogation of 50% of forskolin-stimulated cAMP production. While this effect was observed independently of concomitant exposure to CC, it abrogated the CC-sensitive component (Fig. 3B). These results suggest that the CC-mediated inhibition of cAMP production may be due to mACHR-stimulated influx of calcium and that there may be multiple isotypes of adenyl cyclase activated by forskolin in these cells (described below, Fig. 8).

Lack of Stimulation of Inositol Phosphate Production—The ability of CC to stimulate production of inositol polyphosphates in the A2058 cells was approached with several different experimental designs. These included two different organic solvent separations and an inositol trisphosphate radioreceptor assay. As shown in Fig. 4A, no stimulation of inositol trisphosphate could be detected with the very sensitive radioreceptor assay in the face of a 2-fold stimulation in the CHO m5AChR control cells. Inositol monophosphate, diphosphate, or total inositol polyphosphates were not detectable using organic extraction and anion exchange chromatography methods in response to CC exposure of A2058 cells. Inositol trisphosphate production has been demonstrated previously in these cells in response to autocrine motility factor activation (23). Immunoblot was used to investigate the expression of phospholipase C isozymes in the A2058 cells. Fig. 4B shows the presence of both phospholipase C-β1 and phospholipase C-γ isozymes in lysates of A2058 cells. Since phospholipase C-γ requires phosphorylation for activation, the ability of CC to stimulate tyrosine phosphorylation was tested with 5- and 15-min exposures to CC (Fig. 4C). Very minimal phosphorylation was observed at either time point, suggesting that this isozyme is not a major downstream effector of the m5AChR in these cells.

Stimulation of Arachidonic Acid Release—CC stimulated a concentration-dependent release of arachidonic acid from the A2058-1F5 cells ($EC_{50} = 17.8 \pm 0.1 \text{ μM}$, Fig. 5A). Arachidonic

- **Fig. 1.** Molecular analysis of mAChR subtype. A, A2058 cells contain m5 subtype AChR by PCR amplification. Ethidium bromide-stained gradient polyacrylamide gel shows PCR fragments amplified with odd-numbered AChR selective oligonucleotides (upper panel) from CHO m1, m2, m3, m4, m5, and A2058-1F5 cells (lanes 1-6, respectively). The observed fragment of 1200 kilobases in size is the size expected for amplification of an m5 transcript. Southern hybridization with a PCR fragment amplified using m5-selective oligonucleotides specific to the third cytoplasmic domain confirmed the presence of product only in the m5 and A2058 lanes (lanes 5 and 6, bottom panel). B, A2058 cells do not contain even-numbered AChR message. m2/4 selective oligonucleotides were used to confirm the presence of only one subtype of AChR message in A2058 cells. The upper panel shows the results of PCR amplification using specific primers for m2/4, showing product only in CHO m2 and m4 cells (upper panel, lanes 2 and 4). Southern analysis using an internal oligonucleotide probe selective for m2/4 is shown in the lower panel, confirming the lack of product in the A2058 cells.

- **Fig. 2.** Specific N-[methyl-3H]scopolamine ([3H]NMS) binding to A2058 cell plasma membranes. Plasma membranes were prepared from A2058 cells. Saturation binding analysis was performed as described under "Experimental Procedures" using N-[methyl-3H]scopolamine as the labeled ligand and 10 μM atropine to define nonspecific binding. Data are mean ± S.E., n = 3.
acid release was blocked by co-incubation with atropine, the muscarinic receptor antagonist (Fig. 5B). CC-stimulated arachidonic acid release was first observed within 2 min of agonist addition. A plateau was reached by 10 min and was sustained for 30–60 min (Fig. 5A). All further arachidonic acid release experiments were allowed to proceed for 15 min.

Effect of Protein Kinase C Activation—A2058 cells were incubated with PMA for 15 min, 1, or 24 h to determine the effect of activation and down-regulation of protein kinase C on release of arachidonic acid (Fig. 6). Inclusion of PMA alone for 15 min resulted in a 200% increase in released arachidonic acid which rose to 400%, equivalent to that seen with CC treatment, at a 1-h exposure. An additive effect was observed when cells were exposed to PMA for 1 h followed by CC treatment, suggesting that arachidonic acid release could be stimulated in A2058 cells by protein kinase C, independently of muscarinic receptor-mediated activation. A 24-h exposure of A2058 cells to PMA to down-regulate cellular protein kinase C activity inhibited the CC-stimulated response by approximately 50% and abrogated the PMA-induced arachidonic acid release seen at 1 h, consistent with a requirement for protein kinase C in activation of this response.

Calcium Sensitivity of Arachidonic Acid Release—The mAChR-stimulated release of arachidonic acid from A2058 cells was partially dependent upon extracellular calcium as shown in Fig. 7. Inclusion of the heavy metal cobalt or the Ca<sup>2+</sup> chelator EGTA in the reaction media markedly reduced CC-induced arachidonic acid release (Fig. 7A). Mobilization of calcium through calcium influx can occur through voltage-dependent or independent channels. The voltage-sensitivity of the stimulated arachidonic acid release was further investigated using potassium-mediated cellular depolarization. Exposure of A2058 cells to high K<sup>+</sup> media slightly decreased the release of arachidonic acid without altering the baseline release or ability of atropine to inhibit this response (data not shown).
of increasing concentrations of diltiazem (IC$_{50} = 243 \pm 80 \mu M$) or verapamil (IC$_{50} = 166 \pm 30 \mu M$) resulted in inhibition of CC-mediated arachidonic acid release (Fig. 7B). Nifedipine (not shown) minimally inhibited arachidonic acid release at concentrations below 300 \mu M. These data suggest that the calcium influx required for arachidonic acid release comes from mobilization of calcium through nonvoltage-gated calcium channels.

Effect of Carbachol on Intracellular Calcium Concentrations in A2058 Cells—A2058-1F5 cells were loaded with the calcium-sensitive fluorescent dye, FURA-2, and changes in single cell fluorescence were measured over time (Fig. 8). CC stimulated a rapid increase in intracellular calcium that was attenuated with the addition of a muscarinic receptor antagonist, atropine (Fig. 8A). The relative contribution of extracellular calcium influx was evaluated in Fig. 8B, in which CC was added in the absence of extracellular calcium and induced a transient but rapid rise in intracellular calcium that decayed back to basal levels. After a brief wash period, CC re-exposure resulted in a small rise in intracellular calcium that quickly returned to control levels suggesting that intracellular calcium pools had been depleted almost completely during the first CC application (Fig. 8B). Re-application of calcium-containing media resulted in a large calcium influx that was sustained at a new plateau as long as CC was present. An additional experimental approach was selected to determine if the calcium influx component was down-regulated following a repeat application of CC (Fig. 8C). Intracellular calcium pools were depleted by the addition of CC in calcium-free medium. Sequential addition, removal, readdition, and removal of extracellular calcium in the perfusion buffer caused a concomitant rise and fall of intracellular calcium. At the end of the experiment, a small transient intracellular release of calcium was evoked by CC in calcium-free media, most likely due to partial refilling of cytoplasmic pools during the course of the calcium-containing buffer perfusion. CC induces both a release of calcium from cytoplasmic stores as well as calcium influx in A2058-1F5 cells.

CC Suppresses A2058 Cell Colony Formation in Soft Agar

We have shown previously that activation of the odd-numbered muscarinic receptor species in CHO cells specifically transfected with these receptors results in a morphologic and functional change to a nontumorigenic phenotype indicated by a shift from stellate to a more flattened and fibroblast-like shape (24). The demonstration of this receptor in a human melanoma cell line offered the opportunity to investigate the significance of these observations in an endogenously expressed receptor. When A2058 parental cells or the 1F5 subtype were cultured in monolayer with increasing concentrations of CC, no morphologic changes were detected, nor were changes in monolayer growth potential noted. However, when
CC 0.1–100 μM was included in the culture media of soft agar cultures, a statistically significant decrease in colony formation (p < 0.001) and colony size in soft agar was demonstrated (Fig. 9). The reduction in clonogenic potential suggests a potential suppressor function of this receptor in the melanoma cells.

**DISCUSSION**

The discovery of an endogenous mAChR in the A2058 human melanoma line provides the first demonstration of a novel pattern of mAChR receptor-signal coupling as well as the demonstration of an endogenous mAChR outside of the central nervous system. This receptor couples to release of the arachidonic acid and mobilization of intracellular calcium without measurable production of inositol phosphates or cAMP. Activation of this mAChR results in inhibition of an adenyl cyclase activity that is insensitive to pertussis toxin treatment.
The observed inhibition of production of cAMP by activation of the A2058 m5AChR, instead of the expected stimulation, would not have been predicted on the basis of the receptor subtype. The lack of an even-numbered mAChR or novel muscarinic acetylcholine receptor subtype suggests that the altered signal coupling is a cell-specific process. Mechanisms to explain this different signaling pattern include a different effector protein complement or altered G protein complement in these cells. Even-numbered mAChRs preferentially couple to adenyl cyclase inhibition through Gs and Gi, whereas the odd-numbered mAChRs use the Gq and G11 families to couple to phospholipase C-β (17, 31). The A2058 cells have been shown to have Gαs, Gαi1, and Gia2 and have been shown to couple to inositol trisphosphate production in a pertussis toxin-sensitive fashion (23). The lack of pertussis toxin sensitivity of the inhibition of adenyl cyclase by receptor activation in the A2058 cells further differentiates the receptor-signal coupling of this endogenous mAChR. The increased cAMP produced with pertussis toxin exposure suggests that there is an adenyl cyclase for which a tonic inhibition is relieved by pertussis toxin, releasing adenyl cyclase activity. In a different study, stimulation of m1 receptors expressed at physiologic levels in RAT-1 cells was shown to inhibit adenyl cyclase activity; however, that inhibition was pertussis toxin-sensitive (15). The reverse phenomenon was observed in which overexpressed m4 receptors shifted from inhibition to stimulation of adenyl cyclase by pretreatment with pertussis toxin and blockade of Gi activity (34). In both cases, the aberrant coupling to adenyl cyclase was pertussis toxin-sensitive. Site-directed mutagenesis has identified regions of the third cytoplasmic domains of the m2 and m3 receptors which regulate the pertussis toxin sensitivity and are involved in G protein interactions (35). The inhibition of cyclase activity in A2058 cells after activation of the m5AChR was not abrogated by pertussis toxin exposure, despite availability of the requisite G protein complement and lack of mutation in the critical regions of the third cytoplasmic domain.

The partial inhibition of forskolin-stimulated adenyl cyclase activation by removal of extracellular calcium suggests that there are more than one isozyme of adenyl cyclase isozyme in the A2058 cells. Adenyl cyclase isozymes V and VI are characterized by inhibition in function as a result of increase in intracellular calcium concentration (36). A pertussis toxin-independent, calcium-dependent inhibition of substance K receptor-mediated inhibition of cAMP produced in response to isoproterenol was reported in C6-2B glial cells (37). A similar calcium-dependent inhibition of cAMP accumulation was observed using activation of the bradykinin receptor in NCB-20 plasma membranes (38). Our m5AChR-induced inhibition of adenyl cyclase activity in A2058 cells is calcium influx-sensitive, suggesting that receptor-mediated calcium entry may be the driving inhibitory force.

Another point of disparity between classical receptor-signal coupling of the mAChR and this m5AChR is the activation of...

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**TABLE I**

| Signal produced | Odd (m1, m3, m5) | Even (m2, m4) | A2058 |
|-----------------|-----------------|---------------|-------|
| Arachidonic acid | + | Augmented | + |
| Inositol trisphosphate | + | None | None |
| Calcium influx | + | None | + |
| cAMP | + | Inhibited | Inhibited |

*Dr. S. Aznavoorian, personal communication.*
the phospholipases C to release inositol trisphosphate. The odd-numbered muscarinic receptors have been shown to couple through G proteins to phospholipase C-\(\beta\) (9, 13, 15, 17, 35, 39). In addition, activation of phospholipase C-\(\gamma\) in CHOM5 cells has been demonstrated through muscarinic receptor-mediated and calcium-sensitive phosphorylation (12). A previous report also described an even-numbered mAChR coupling to phosphoinositide turnover in a pertussis toxin-sensitive pathway (18). Both organic separation and anion chromatography, and an inositol trisphosphate radioreceptor assay failed to demonstrate a rise in inositol phosphates in response to CC treatment of A2058 cells where CHOM5 cell controls were positive. It is possible that the production of inositol trisphosphate was below the level of detection of these assay systems; however, this is unlikely as the measurement was approached in three independent ways and the radioreceptor assay has picogram sensitivity.

Western immunoblot demonstrated the presence of phospholipase C-\(\beta\) and phospholipase C-\(\gamma\) isozymes in A2058 cells. The relative lack of phosphorylation of phospholipase C-\(\gamma\) after exposure to CC for 5 or 15 min argues for it not being coupled to the m5AChR in these cells. This further underscores the different coupling effector system in these cells, as activation of phospholipase C-\(\gamma\) in CHO cells stably transfected with the m5AChR has been demonstrated (12). The observed release of internal calcium under calcium-free conditions argues for the presence of inositol trisphosphate or alternative internal calcium-mobilizing, inositol trisphosphate-independent mechanisms.

The A2058 m5AChR coupled to release of arachidonic acid and mobilization of calcium influx. Arachidonic acid release proceeded with an \(EC_{50}\) similar to that previously described for the transfected m5 receptor (10, 24, 32). The requirement for extracellular calcium was demonstrated by the inhibition of the CC response by inclusion of EGTA in the reaction buffer and increase in CC-stimulated arachidonic acid release in the presence of calcium. The molecular mechanism for calcium in activation of phospholipase A2 has been identified (30, 40–42). Calcium is involved in cPLA2 translocation, phosphorylation, and activation by mitogen-activated protein kinase. mAChR have been shown to regulate influx of calcium through nonvoltage-gated calcium channels, specifically, the receptor-operated channels (10–12, 14, 17, 30, 43). The A2058 m5AChR stimulates receptor-operated calcium influx as shown by the ability of atropine to block influx. Last, the A2058 m5AChR follows previously described patterns in arachidonic acid release in its sensitivity to protein kinase C (10, 33). Activation of protein kinase C through phorbol ester exposure results in activation of arachidonic acid release itself and augments the CC-stimulated effects in the A2058 cells.

Activation of the A2058 m5AChR with CC resulted in a receptor-specific inhibition in A2058 cell clonogenic capacity in soft agar assays. Anchoragel-independent growth in culture is a marker for tumorigenicity. We have previously demonstrated the phenomenon of receptor-mediated anti-oncogenesis using CHOM5 cells (24). Exposure of CHOM5 cells to CC in soft agar cultures also resulted in inhibition of anchorage-independent growth. The reduction in tumorigenicity in CC-activated CHOM5 cells was confirmed further in nude mouse xenograft experiments. Treatment of the inoculation site with CC markedly reduced the tumor incidence and those tumors that did initiate had an extremely low growth rate. Inclusion of atropine reversed the effects of CC yielding a full tumor incidence and exponential tumor growth. Similarly, an anti-proliferative effect of CC-activation of transfected m1 receptors has been documented in A9 cells (8, 44). The parallel results of reduction in soft agar colony formation with activation of the m5 receptor in the A2058 cells suggests that the A2058 m5AChR may have an anti-oncogenic function.

The classical muscarinic receptor signal transduction coupling has followed receptor-specific patterns as described in Table I. These signaling patterns have been confirmed using endogenous systems and transfected receptor models and have yielded identical results. To date, there has been no endogenous m5AChR available to confirm the signaling pattern previously described using stable transfectants (10, 11, 17). This endogenous A2058 melanoma cell m5AChR is a low abundance, high affinity receptor with novel signal effector coupling. The unique signal coupling demonstrates the importance of the host cell in establishing permissive downstream effector signal transduction and offers new insight into the biology and biochemistry of the muscarinic receptor system.

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