The M1 Receptor Is Required for Muscarinic Activation of Mitogen-activated Protein (MAP) Kinase in Murine Cerebral Cortical Neurons

Susan E. Hamilton and Neil M. Nathanson

From the Department of Pharmacology, University of Washington School of Medicine, Seattle, Washington 98195-7750

Muscarnic acetylcholine receptors (mAChR) are the predominant cholinergic receptors in the central nervous system where they are involved in learning and memory, epileptic seizures, and processing the amyloid precursor protein. The M1 receptor is the predominant mAChR subtype in the cortex and hippocampus. Although the five mAChR fall into two broad functional groups, all five subtypes, when expressed in recombinant systems, can activate the mitogen-activated protein kinase (MAPK) pathway. The MAPK pathway has been implicated in learning and memory, amyloid protein processing, and neuronal plasticity. We used M1 knock-out mice to determine the role of this receptor subtype in signal transduction in the mouse forebrain. In primary cortical cultures from mice lacking the M1 mAChR, agonist-stimulated phosphoinositide hydrolysis was reduced by more than 60% compared with cultures from wild type mice. Although muscarinic agonists induced robust activation of MAPK in cortical cultures from wild type mice, mAChR-mediated activation of MAPK was virtually absent in cultures from M1-deficient mice. These results indicate that the M1 mAChR is the major subtype that mediates activation of phospholipase C and MAPK in mouse forebrain.

Muscarnic acetylcholine receptors (mAChR) are the predominant cholinergic receptors in the central nervous system where they are involved in learning and memory (1), in epileptic seizures (2), and in processing the amyloid precursor protein (3, 4). Of the five subtypes of these seven transmembrane, G protein-coupled receptors, M1 is found in greatest abundance in the cortex and hippocampus where it constitutes 40–50% of the total mAChR (5). The M1, M3, and M5 subtypes preferentially couple via the Galpha11 protein family to activate phospholipase C (PLC), stimulating phosphoinositide (PI) hydrolysis (6). The resulting products, diacylglycerol and inositol trisphosphate, activate PKC and trigger a release of intracellular calcium, respectively. These events initiate an overlapping network of signals, including the activation of mitogen-activated protein kinase (MAPK) (7). M2 and M4 preferentially couple via G proteins to inhibit adenylyl cyclase activity but in some cell types can also activate PLC (8, 9). These subtypes can also activate MAPK by direct G protein beta subunit-mediated mechanisms (10) and potentially also via activation of PKC.

The MAP kinases ERK1 and ERK2 (p44 and p42) are known to play a pivotal role in signaling events in the brain where they are involved in neuronal plasticity (11), neuronal survival (12), and processing the amyloid precursor protein (13, 14). They play a crucial role in learning and memory, including long term potentiation (15, 16) and long term spatial memory (17). In addition to activation by G protein-coupled receptors, the ERKs can be stimulated by growth factors signaling through receptor tyrosine kinases. Thus these kinases are poised at a critical position allowing cross-talk between a variety of signal transduction pathways.

Although the functional properties of the individual mAChR subtypes have been well characterized in recombinant systems, the analysis of endogenous mAChR function in cells and tissues expressing multiple subtypes of mAChR is complicated by the lack of subtype-specific agonists and overlapping selectivity of antagonists. In addition, the apparent affinity of a putative subtype-selective antagonist can vary dramatically depending on membrane composition and lipid environment (18), so that the same receptor polypeptide can potentially exhibit different ligand affinities in different cell types. We have previously shown that in mice with a targeted deletion of the gene encoding the M1 receptor, mAChR-mediated suppression of the M current potassium channel is completely lost in sympathetic neurons (19), whereas mAChR-mediated suppression of the M current in hippocampal neurons is unaltered (20). Thus, a given subtype of mAChR may not couple to the same functional pathway in different cell types. In this paper, we use mice lacking the M1 receptor to demonstrate that M1 is the main receptor subtype responsible for activation of PLC and MAPK in cultured cerebral cortical neurons.

EXPERIMENTAL PROCEDURES

Materials—Neurobasal medium, B-27 supplement, and penicillin-streptomycin were obtained from Life Technologies, Inc. Carbamylcholine chloride (carbachol), cytosine-beta-D-arabinoside, phorbol 12-myristate 13-acetate (PMA), tetrodotoxin, atropine sulfate, and papain came from Sigma. Research Biochemicals International was the source of oxotremorine methiodide (oxo), (D)-2-amino-5-phosphonopentanoic acid (APV), nimodipine, and trans-1S,3R-1-amino-1,3-cyclopentanedicarboxylic acid (ACPD). ICN Biomedicals Inc. supplied 6-cyano-7-nitroquinoxaline-2,3-dione (CNPX). Myo-[2-3H]inositol and ECL reagents came from Amersham Pharmacia Biotech, poly-D-lysine hydrobromide (high molecular weight) came from Collaborative Biomedical Products, phospho-p44/42 MAPK antibody came from New England Biolabs, monoclonal

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anti-MAPK (ERK1 + ERK2) antibody came from Zymed Laboratories Inc., Immobilon-P membrane came from Millipore, and ion exchange resin AG 1-X8 100–200 mesh (formate form) came from Bio-Rad.

**Immunoblot Analysis for Detection of M1 mACHr**—Membranes were prepared from mouse heart, cerebellum, and forebrain as described by Liuet al. (21). Dissected mouse tissues were placed in phosphate-buffered saline (PBS) containing protease inhibitors, homogenized on ice 25 times in a glass-class homogenizer, and centrifuged at 36 × g for 15 min to remove debris. The supernatant was centrifuged at 8000 × g for 15 min to pellet membranes. After determination of protein concentration, 55 μg of membrane protein was solubilized in an SDS sample buffer containing 8 M urea and loaded onto a 3.5% stacking SDS-polyacrylamide gel and a 7% separating SDS-polyacrylamide gel, each containing 4 μl urea. Proteins were transferred to Immobilon-P membrane, and immunoblot analysis was carried out as described by McKinsson and Nathanson (22) using affinity-purified polyclonal antianti-M1 antibody directed against the third cytoplasmic loop of the mouse M1 (19).

**Primary Cortical Cell Culture**—Primary cortical cultures were prepared as described by Chan et al. (23). Briefly, the cerebral cortices of newborn mouse brains were digested with papain for 20 min at 37 °C. The rinsed tissue was then triturated 15 times and allowed to settle. Supernatants were saved, and the trituration was repeated on remaining tissue. Supernatants were combined, and cortical neurons were seeded on plates treated with polylysine for a minimum of 2 h. Neurons for PI assays and determination of MAPK activation were plated in 24-well (0.15 × 106 cells/well) and 6-well (1 × 106 cells/well) plates, respectively. Cells were grown in neurobasal medium containing B-27 supplement, glutamine (1 mM), penicillin (0.05 units/ml), and streptomycin (0.05 μg/ml) and maintained at 37 °C in 5% CO2. Medium was changed one and three days after plating and contained 3.6 μM cytosine-β-D-arabinoside to retard glial growth.

**Quantitative Immunoprecipitation Analysis of M1 Expression**—The expression of the M1 mACHr was determined by immunoprecipitation analysis using a polyclonal antibody specific for the M1 mACHr as described (19).

**Phosphoinositide Hydrolysis Assays**—After six to eight days in culture, culture media was measured as described (24, 25). In brief, cells were incubated overnight in culture medium containing 1 μCi/ml myo-[2-3H]inositol (18.3 Ci/mmol). Cells were washed and cultured 30 min at 37 °C with physiological saline solution (118 mM NaCl, 4.7 mM KCl, 3 mM CaCl2, 1.2 mM KH2PO4, 10 mM glucose, 0.5 mM EDTA, 20 mM HEPES, pH 7.4) containing 10 mM LiCl. Carbaclo or ACPD was added for 15 min before the reaction was terminated with ice-cold methanol. Total inositol phosphates were quantified using ion exchange chromatography. Each drug condition was performed in quadruplicate for each of four cultures/genotype.

**Determination of MAPK Activation**—MAPK activation was performed on cultures seven to 10 days after plating. Two h before stimulation, cultures were treated with tetrodotoxin (1 μM), CNQX (40 μM), APV (100 μM), and nifedipine (5 μM) to decrease endogenous synaptic activity (26). Cells were stimulated for the intervals indicated with oxo. Medium was aspirated, and cells were incubated overnight in culture medium containing 1 μCi/ml [2-3H]inositol (18.3 Ci/mmol). Cells were washed and homogenized on ice 25 times in a glass-class homogenizer, and centrifuged at 36 × g for 15 min to remove debris. The supernatant was centrifuged at 8000 × g for 15 min to pellet membranes. After determination of protein concentration, 55 μg of membrane protein was solubilized in an SDS sample buffer containing 8 M urea and loaded onto a 3.5% stacking SDS-polyacrylamide gel and a 7% separating SDS-polyacrylamide gel, each containing 4 μl urea. Proteins were transferred to Immobilon-P membrane, and immunoblot analysis was carried out as described by McKinsson and Nathanson (22) using affinity-purified polyclonal anti-M1 antibody directed against the third cytoplasmic loop of the mouse M1 (19).

**Results**

**Functional M1 mACHr Protein Fragments Are Not Reconstituted in M1-deficient Mice**—Previously, we showed that the M1-KO mice do not express detectable M1 protein as determined by immunoprecipitation of radioligand-labeled, digitonin-solubilized receptors (19). Recent work has shown that fragments of the mACHr can interact to form a reconstituted functional receptor. The interaction between receptor fragments containing transmembrane domains I–V and fragments containing transmembrane VI–VII depends on the presence of the long third intracellular loop, and chimeric receptors containing these separate domains exhibited agonist-induced functional activity (27, 28, 29). Because our targeting construct used to generate M1-KO mice lacked the nucleotides coding for the first 55 amino acids of the M1 receptor, it would be possible for protein synthesis to initiate at a downstream methionine, resulting in the expression of a truncated polypeptide containing most of the transmembrane domains as well as the third cytoplasmic loop. If this truncated protein were indeed synthesized, it could interact with one of the remaining mACHr polypeptides to produce a protein exhibiting the functional coupling of the M1 receptor but would not necessarily be stable to detergent solubilization and subsequent detection by immunoprecipitation. To determine whether this possibility was occurring, we performed immunoblot analysis using an antisera prepared against the third cytoplasmic loop of the M1 receptor to ensure that this portion of M1 was absent in our M1-KO mice. In addition to mouse tissues, protein from the mouse Y1 adrenal cell line and Y1 cells stably transfected with mouse M1 (Y1-M1) under an inducible promoter (24) were analyzed simultaneously. A strong signal was detected in the WT forebrain, which was significantly reduced in forebrain from heterozygote mice (Fig. 1). As expected from the negligible expression of the M1 receptor in cerebellum and heart, no signal was detected in these tissues. Immunoreactivity to M1 was not detectable in any of the M1-KO tissues. The Y1-M1 cells express two immunoreactive proteins, the lower corresponding to the band in the M1-WT and WT forebrain samples with an apparent molecular mass of 60 kDa. These results, combined with our previous data (19), indicate that a polypeptide containing the functionally vital third cytoplasmic loop of the M1 receptor is completely absent in the M1-KO mice and that there most likely is no ectopic translation of a truncated protein.

**Decreased mACHR-mediated Activation of PLC in M1-deficient Cortical Cultures**—To measure the effects of inactivating the M1 receptor on mACHR-mediated activation of PLC, we measured the stimulation of PI hydrolysis in primary cortical cultures derived from WT and M1-KO mice in response to the muscarinic agonist carbaclo. The mean maximum increase in inositol phosphates observed with WT cultures was 250% above basal level (Fig. 2A). In M1-KO cultures this maximal level was reduced by a factor of 2.6 with a mean stimulation of 95% above basal.
MAPK Activation in M₁-deficient Cortical Neurons

FIG. 2. Muscarinic receptor-mediated phosphoinositide hydrolysis in cortical cultures from WT and M₁-KO mice. Primary cortical cultures from WT and M₁-KO mice were incubated with myo-[2-³H]inositol overnight and subjected to 15 min stimulations with the mACHR agonist, carbachol (A), or the metabotropic glutamate receptor agonist, ACPD (B) at the concentrations indicated. Results are expressed as percent stimulation above basal and represent the mean ± S.E. of four sets of cultures analyzed in quadruplicate at each concentration.

We previously showed by quantitative immunoprecipitation analyses that the level of the remaining mACHR subtypes was unaltered in the forebrain of M₁-KO mice (19). In addition to M₁, M₃ couples via the Gγ₁₁-family and is present in significant amounts in the forebrain. To ensure that levels of M₃ were not altered in cortical cultures, we carried out quantitative immunoprecipitation analyses with an anti-M₃-specific antibody. The levels of M₃ were similar in cortical cultures prepared from WT (88 ± 17 fm/mg protein) and M₁-KO (73 ± 18 fm/mg protein) mice (mean ± S.E.; n = 4).

Metabotropic glutamate receptors also activate PLC via Gγ₁₁ proteins. ACPD, a selective agonist for the metabotropic glutamate receptor, was used as a positive control to ensure that no general defect in G protein or PLC function existed. Similar levels of maximal activation by ACPD were obtained from WT and M₁-KO cortical cultures (Fig. 2B), indicating that the PLC signaling pathway downstream of the M₁ mACHR is normal in M₁-KO mice.

Decreased mACHR-mediated MAP Kinase Activation in M₁-deficient Cortical Cultures—Dual phosphorylation of the MAP kinases ERK1 and ERK2 on Thr-202 and Tyr-204 by MAPK kinase results in their activation; the detection of this dual phosphorylation using a dual-phospho-specific antibody is a widely used, sensitive method for the quantitation of the activation of MAPK in cultured cells and intact tissues (13, 15–17). We performed SDS-polyacrylamide gel electrophoresis and immunoblot analysis using antibodies specific to the activated (dually phosphorylated) forms of ERK to measure stimulation of MAPK in primary cortical cultures from WT and M₁-KO mice. In WT cultures the time course of treatment with the agonist oxo showed maximal levels of phospho-ERK within 5–10 min of agonist stimulation with a return to basal level by 30 min (Fig. 3A, a). This blot was stripped and reprobed with an antibody to the non-phosphorylated forms of ERK to ensure that differences in band intensity observed with phospho-ERK antibodies were not due to differences in protein loading (Fig. 3A, b). Immunoblot analyses comparing stimulation of WT and M₁-KO cultures showed a robust activation of MAPK by oxo in WT cortical neurons (Fig. 3B, a) and low to nonexistent activation in the M₁-KO cultures (Fig. 3C, a). Quantitative analysis of the immunoblots showed that WT cultures treated with oxo exhibited a mean stimulation of >300% above basal compared with 31% for the M₁-KO cultures (n = 5 for each genotype; Fig. 4). This activation is decreased by >75% when cultures are pretreated with the muscarinic-specific antagonist atropine, indicating that the effects of oxo are due to mACHR activation. PMA, a direct activator of PKC, induced a similar level of MAPK activation in WT (1050%) and M₁-KO cultures (890%), respectively, indicating that the signaling pathway coupling stimulation of PKC to activation of the MAPK pathway is unaltered in M₁-deficient neurons.

DISCUSSION

A growing number of studies show that functionally significant intermolecular interactions between muscarinic receptors can occur and that the third cytoplasmic loop is vital for these
potential heteromolecular interactions. In the original construct designed to ablate the M1 gene product by homologous recombination, nucleotides coding for the N terminus and first transmembrane domain were deleted (19). However, the nucleotide sequence for >85% of the coding sequence, including the third cytoplasmic loop, is still present in these mice. To ensure that this portion of the receptor was not expressed, we performed immunoblot analyses using an M1-specific antibody raised against the third cytoplasmic loop of M1 (amino acids 233–332). Our results confirm that this key region of the receptor is significantly reduced in the forebrain of the M1-KO mice and is not detected in the M1-KO mice.

Studies using recombinant M1 mAChR show that it couples via the Gq/11 protein family to the activation of PLC (24, 30). The M1 receptor constitutes approximately half of the total mAChR in adult mouse forebrain (19). In primary cortical cultures from newborn M1-KO mice, agonist-stimulated PI hydrolysis was reduced by >60% compared with WT cultures. Thus, the M1 receptor is the predominant mAChR subtype responsible for PLC activation in mouse cortical neurons. Despite the loss of M1, there was still significant carbachol-stimulated PLC activation in mouse cortical neurons. Deletion of the second cytoplasmic loop of M1 also reduced agonist-stimulated PLC activation in mouse cortical neurons. Our results confirm that this key region of the receptor is still present in these mice. To ensure this, the nucleotide sequence for transmembrane domain were deleted (19). However, the nucleotide sequence for G0/11, family from their receptors, inhibited 70% of this agonist-induced activation, suggesting that either ectopic coupling of M1 to G0/11 proteins or signal transduction downstream of M1 also contributes to the muscarinic agonist activation in this neuronal cell line.

MAPK activation in our WT primary cortical cultures ranged from 180 to 540% over basal values, similar to the values reported in rat cortical cultures (Fig. 3A, Ref. 16). The maximal MAPK activation occurred in our WT mouse cultures within 5–10 min of agonist stimulation and rapidly returned to baseline, in contrast to the more persistent (30–60 min) activation reported for rat cultures (16). This may be due to differences in species or tissue culture conditions. In M1-KO cultures the range of MAPK stimulation by agonist was reduced to ~70 to 120%. The average magnitude of stimulation decreased from 320% over basal in cultures from WT mice to 31% in the cultures from M1-KO mice. Thus M1 is the mAChR responsible for coupling to ERK activation in our primary cortical neuron culture system. The M1 receptor is present in similar amounts in our WT and KO cultures and most likely accounts for the agonist-induced PLC activation remaining in the M1-KO cultures. The much greater impairment in MAPK activation compared with PLC activation observed in the M1-KO cultures suggests that the M3 receptor, although still able to evoke significant activation of PLC, is relatively ineffective in mediating activation of MAPK in these neuronal cultures. PMA-induced activation of MAPK was similar in WT and M1-KO cultures indicating that signaling downstream of PCK is unaltered in mice lacking M1 mAChR.

In summary, these results show that there are significant defects in mAChR-mediated activation of PLC and MAPK in the forebrains of mice lacking the M1 receptor. Both the M1 and M3 receptors couple to members of the G0/11 family of G proteins, although it is not known if there are subtle differences in the specificity of the two receptors for either α or βy subunits; the results here suggest that the signaling pathways activated by the two receptors in cortical neurons appear to be different. The M1-KO mice will be useful in determining the PKC-dependent and PKC-independent pathways by which mAChR mediate secretion of soluble amyloid precursor protein. In addition, these mice should be useful in determining the relationship between M1 mAChR, MAPK activation, long-term potentiation, and learning and memory. Indeed, preliminary data indicate that mice lacking the M1 mAChR exhibit defects in the consolidation of hippocampus-dependent learning and show a deficit in long term potentiation (33).

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