Function Activity of the M$_2$ and M$_4$ Receptor Subtypes in the Spinal Cord Studied with Muscarinic Acetylcholine Receptor Knockout Mice

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ABSTRACT

Stimulation of spinal muscarinic acetylcholine receptors (mACHRs) produces potent analgesia. Both M$_2$ and M$_4$ mACHRs are coupled to similar G proteins (G$_{o/}$ family) and play a critical role in the analgesic action of mACHR agonists. To determine the relative contribution of M$_2$ and M$_4$ subtypes to activation of G$_{o/}$ proteins in the spinal cord, we examined the receptor-mediated guanosine 5'-O-(3-[35S]thio)triphosphate ([35S]GTPyS) binding in M$_2$ and M$_4$ subtype knockout (KO) mice. Basal [35S]GTPyS binding in the spinal cord was similar in the wild-type controls, M$_2$ and M$_4$ single-KO, and M$_2$/M$_4$ double-KO mice. The spinal [35S]GTPyS binding stimulated by either muscarine or oxotremorine-M was not significantly different among three groups of wild-type mouse strains. In M$_2$ single-KO and M$_2$/M$_4$ double-KO mice, the agonist-stimulated [35S]GTPyS binding was completely abolished in the spinal cord. Furthermore, the agonist-stimulated [35S]GTPyS binding in the spinal cord of M$_4$ single-KO mice was significantly reduced (~15%), compared with that in wild-type controls. On the other hand, the spinal [35S]GTPyS binding stimulated by a μ-opioid agonist was not significantly different between wild-type and M$_2$ and M$_4$ KO mice. This study provides complementary new evidence that M$_2$ is the most predominant mACHR subtype coupled to the G$_{o/}$ proteins in the spinal cord. Furthermore, these data suggest that a small but functionally significant population of M$_4$ receptors exists in the mouse spinal cord. The functional activity of these M$_4$ receptors seems to require the presence of M$_2$ receptors.

The cholinergic system and muscarinic acetylcholine receptors (mACHRs) in the dorsal horn of the spinal cord are important for regulation of different physiological functions including nociception. In this regard, intrathecal administration of muscarinic receptor agonists or acetylcholinesterase inhibitors produces potent analgesia in both animals and humans (Iwamoto and Marion, 1993; Naguib and Yaksh, 1994, 1997; Hood et al., 1997). The analgesic effect produced by muscarinic receptor agonists or acetylcholinesterase inhibitors is blocked by the mACHR antagonist atropine (Naguib and Yaksh, 1994). Furthermore, spinal acetylcholine and mACHRs are involved in the analgesic action produced by morphine and α$_2$-adrenergic receptor agonists (Pan et al., 1999; Chen and Pan, 2001). It has been shown that neurons and nerve terminals expressing choline acetyltransferase and acetylcholinesterase (enzymes for acetylcholine synthesis and degradation) are located in the spinal dorsal horn (Ribeiro-da-Silva and Cuello, 1990; Wetts and Vaughn, 1994). Autoradiographic studies have demonstrated that the highest density of mACHRs in the spinal cord is distributed in the superficial laminae in both rats and humans (Yamamura et al., 1983; Villiger and Faull, 1985; Maher et al., 2001).

Molecular cloning studies have revealed the existence of five molecularly distinct mACHR subtypes referred to as M$_1$ to M$_5$ (Wess, 1996; Caulfield and Birdsal, 1998). The M$_1$ to M$_5$ mACHRs are prototypical members of the superfamily of G protein-coupled receptors. The M$_1$, M$_3$, and M$_5$ receptor subtypes couple preferentially to the G$_{q/11}$ protein, whereas the M$_2$ and M$_4$ receptors are preferentially coupled to G$_{o/}$ proteins (Caulfield, 1993; Felder, 1995). In rodents, both the M$_2$ and M$_4$ subtypes that are coupled to the pertussis toxin-sensitive G$_{o/}$ proteins have been implicated in the inhibitory

**ABBREVIATIONS:** mACHR, muscarinic acetylcholine receptor; KO, knockout; [35S]GTPyS, guanosine 5'-O-(3-[35S]thio)triphosphate; GDP, guanosine diphosphate; DAMGO, (d-Ala$_2$, N-Me-Phe$_4$,Gly-ol$^{-}\$)-enkephalin; GTP, guanosine triphosphate; QNB, quinuclidinyl benzilate; WT, wild-type.
effect of mAChR agonists on nociception and spinal dorsal horn neurons (Ellis et al., 1999; Gomeza et al., 1999a; Dutta-
roy et al., 2002; Chen and Pan, 2004). Recent studies in mutant mouse lines deficient in M2 and M4 mAChrRs [knock-
out (KO) mice] indicate that the spinal analgesic effect pro-
duced by mAChR agonists is mediated by M2 and M4 sub-
types (Gomeza et al., 1999a; Duttaroy et al., 2002). Although
behavioral experiments suggest the presence of the M4 subtype in the spinal cord of mice, this subtype could not be
directly demonstrated in the mouse spinal cord using immu-
nocytocchemical techniques (Duttaroy et al., 2002).

Materials and Methods

Animals. The generation of M2 and M4 mAChr single-KO and
M2/M4 double-KO mice has been described previously (Gomeza et al.,
1999a; Duttaroy et al., 2002). The genetic background of these mice was
M2 single-KO, 129S1/C57BL/6J hybrids; M4 single-KO, 129SvEv/C57BL/6J hybrids; and M2/M4 double-KO, 129SvEv/C57BL/6J hybrids.
Wild-type mice of the same genetic background as M2, M4, and
M2/M4 KO mice served as controls. To generate mice deficient in both
M2 and M4 mAChrRs, homozygous M4 receptor KO mice were mated
with homozygous M2 receptor KO mice (Duttaroy et al., 2002). The
resulting F1 compound heterozygotes were then intercrossed to gen-
erate M2/M4 double-KO mice (F2 generation). In parallel, the wild-
type F2 mice were interbred to obtain wild-type control mice. Thus,
both M2/M4 double-KO mice and the corresponding wild-type control
couple had an equivalent genetic background [129/Sv1 (25%) 
× 129SvEv (25%) × C57BL/6J (50%)]. Mice were maintained by intemating
homozygous KO and the corresponding wild-type strains. All ani-
mals used for this study were amplified by Taconic Farms (German-
town, NY), where they were raised and housed under identical con-
ditions. Mouse genotyping was carried out by Southern blotting and
polymerase chain reaction analysis of mouse-tail DNA, as described
previously (Gomeza et al., 1999a; Duttaroy et al., 2002). All experi-
ments were performed using male mice (5–6 weeks old). In all experi-
ments, KO mice and age-matched wild-type mice of the
proper genetic background were performed in parallel. Mice were first
anesthetized with 4 to 5% halothane, and the whole spinal cord
was rapidly removed by hydraulic extrusion. The spinal cord tissue
was immediately frozen in 2-methylbutane (Sigma-Aldrich, St.
Louis, MO) in the presence of dry ice and stored at –80°C. The experimen-
tal protocols and procedures were approved by the Animal Care
and Use Committee of the Pennsylvania State University Col-
lege of Medicine and conformed to the guidelines of the National
Institutes of Health Guide for the Care and Use of Laboratory Ani-
males.

Muscarinic Agonist-Stimulated [35S]GTPγS Binding in Spi-
nal Membranes. The [35S]GTPγS binding stimulated by muscarinic agonists was conducted to quantify functional muscarinic receptors in the spinal cord, as described previously (Chen and Pan, 2003). The
[35S]GTPγS binding assay is based on the principle that the inactive
state of the G protein α subunit has a relatively high affinity for GDP
over GTP, whereas activation of a G protein-coupled receptor by its
agonist shifts the α subunit into a higher affinity for GTP versus
GDP. The [35S]GTPγS is a hydrolysis-resistant form of GTP, and the
degree to which an agonist stimulates [35S]GTPγS binding can be
quantified in tissue membranes. Unlike conventional radioligand
binding techniques, the agonist-stimulated [35S]GTPγS binding can
provide functional information about the receptor (i.e., coupling of
G proteins to activated receptors) (Lazareno, 1999; Sim-Selley
and Childers, 2002). Two structurally different nonselective mAChr
agonists, muscarine and oxotremorine-M, were used to activate
mAChrRs and stimulate spinal cord [35S]GTPγS binding. To harvest
adequate amounts of membrane proteins, the spinal cords from two
three mice in the M2/M4 KO and corresponding wild-type groups
were used in each binding experiment. The tissue was homogenized
and disrupted by sonication in ice-cold 50 mM Tris buffer containing
3 mM MgCl2 and 1 mM EGTA (pH 7.4). The homogenate was then
centrifuged at 500g for 10 min at 4°C. The pellet was discarded,
and the supernatant was centrifuged at 48,000g for 30 min at 4°C.
The pellet was resuspended in fresh assay buffer (50 mM Tris, 3 mM
MgCl2, 100 mM NaCl, and 0.2 mM EGTA, pH 7.7) and was cen-
trifuged again as described above. The final pellet was resuspended
in 2 ml of the assay buffer and sonicated for 5 s. The protein content
was measured using the Bio-Rad protein assay kit (Bio-Rad, Her-
cules, CA).

The concentration-dependent effect of agonist-stimulated [35S]-
GTPγS binding was determined by incubating the tissue homoge-
uate with 0.01 to 100 μM muscarine or 0.005 to 25 μM oxotremo-
rine-M, 30 μM GDP, 0.05 nM [35S]GTPγS, 4 μM/ml adenosine
deaminase, 20 μg of protein, and assay buffer in a final volume of
1 ml (in duplicates). The basal binding was determined in the presence
of GDP and absence of muscarinic agonists, and nonspecific binding
was assessed in the presence of 10 μM unlabeled GTPγS. The con-
centrations of muscarine and oxotremorine-M were chosen based
on previous experiments (Chen and Pan, 2003) and pilot studies. After
incubation at 30°C for 1 h, the reaction was terminated by filtration
through Whatman GF/B filters (Whatman, Clifton, NJ) on a cell
harvester with cold 50 mM Tris buffer (pH 7.4). Radioactivity was
determined by immersion of filters in scintillation fluid, incubated
for 2 h at room temperature, and counted by an LS6500 liquid
scintillation counter (Beckman Coulter, Inc., Fullerton, CA). Nonlin-
ear regression analyses of concentration-effect curves were per-
formed using Prism (GraphPad Software Inc., San Diego, CA).

To determine whether the reduced [35S]GTPγS binding stimulated
by muscarinic agonists in KO mice was due to altered G protein
levels or agonist-induced G protein coupling, we repeated agonist-
stimulated [35S]GTPγS binding using a selective opioid agonist,

\[ \text{o-Ac, NMe-Phe}^4, \text{Gly} \cdot \text{ol}^5 \] - enkephalin (DAMGO), as the control. The
DAMGO concentration used was 0.001 to 10 μM (Chen et al., 2002).

[35S]GTPγS binding assays and data calculation were performed
as described above. GDP, muscarine, oxotremorine-M, atropine,
DAMGO, and adenosine deaminase were obtained from Sigma-
Aldrich. GTPγS was purchased from Roche Diagnostics (Indianap-
olis, IN). All other reagent grade chemicals were obtained from Sigma-
Aldrich.

Quantification of M2 Receptors Using Immunoprecipita-
tion and Radioactive Binding Assay. Because we observed that
muscarinic agonist-stimulated [35S]GTPγS binding was signifi-
cantly reduced in M2 KO mice (see Results), the following immunoprepri-
tation and radioligand binding experiments were performed to
determine whether the M2 receptor levels are reduced in the spinal
cord of M2 KO mice. An M2 subtype-specific antiserum was raised
against a nonconserved region of the third cytoplasmic loop of the
mouse M2 receptor protein (Gomeza et al., 1999a,b). The mice were
decapitated, and the spinal cords were quickly harvested. Two spinal
cords were used to obtain adequate membrane proteins for each
assay. The spinal tissue was homogenized in ice-cold 0.32 M sucrose
in 5 mM Tris-HCl buffer containing 1 mM phenylmethanesulfonyl
fluoride. The homogenate was centrifuged at 500g for 10 min at 4°C.
The pellet was discarded, and the supernatant was centrifuged again at 48,000g for 20 min at 4°C. Then the pellet was resuspended in assay buffer (25 mM phosphate buffer containing 5 mM MgCl₂ and 1 mM phenylmethanesulfonyl fluoride, pH 7.4) and was centrifuged as described above. The final pellet was resuspended in 3 ml of the same buffer and disrupted by sonication for 5 s. The membrane protein was subsequently incubated for 1 h with 2 nM [³H]quinuclidinyl benzilate ([³H]QNB), the nonselective muscarinic antagonist (42 Ci/mol; PerkinElmer Life and Analytical Sciences), in the final volume of 1 ml. After washing thoroughly, the labeled membranes were solubilized with 1% digitonin and followed by immunoprecipitation of solubilized [³H]QNB-labeled receptors with the M₂ subtype-selective antiserum, as described previously (Gomez et al., 1999a,b). Radioactivity was quantified using a Beckman LS6500 scintillation counter. Data were analyzed using the GraphPad Prism program.

Data Analysis. Data are presented as means ± S.E.M. The saturation binding data were processed and fit using nonlinear regression analysis (GraphPad Prism software). For computation of EC₅₀ values, [³⁵S]GTPγS binding data were converted to the percentage of stimulation by muscarine and oxotremorine based on the following calculation: [(net stimulated binding/maximal net stimulated binding)] × 100%. The EC₅₀ values of muscarine and oxotremorine-M were determined by nonlinear regression analyses of the concentration-response curves using GraphPad Prism software. The binding data between matched pairs were analyzed using the Wilcoxon rank sum test. For comparisons in more than two groups, the Kruskal-Wallis test was performed followed by the Mann-Whitney U post hoc test. P < 0.05 was considered statistically significant.

Results

Basal [³⁵S]GTPγS Binding in the Spinal Cord. The basal [³⁵S]GTPγS binding, in the absence of muscarinic receptor agonists, was almost identical in the spinal cord from the three wild-type mouse strains (Fig. 1). Basal [³⁵S]GTPγS levels were also similar in spinal cord tissues in the muscarine and oxotremorine control groups. Furthermore, the basal [³⁵S]GTPγS binding in the spinal cord from M₂ single-KO, M₄ single-KO, and M₂/M₄ double-KO mice was not significantly different from the corresponding wild-type controls (Fig. 1).

Spinal [³⁵S]GTPγS Binding Stimulated by Muscarinic Agonists in Wild-Type and KO Mice. The two nonselective mAChR agonists muscarine and oxotremorine-M produced a significant and concentration-dependent increase in [³⁵S]GTPγS binding in the spinal cord of all three strains of wild-type mice (Figs. 2 and 3). In the presence of 10 µM atropine, muscarine and oxotremorine-M failed to cause a detectable increase in the spinal [³⁵S]GTPγS binding in wild-type mice (data not shown). Both 50 to 100 µM muscarine and 10 to 25 µM oxotremorine-M produced saturable [³⁵S]GTPγS binding in the spinal cord, the magnitude of which was comparable in three different wild-type strains (Figs. 2 and 3). The EC₅₀ values for muscarine in the M₂, M₄, and M₂/M₄ wild-type groups were 3.54 ± 0.12, 3.61 ± 0.12, and 3.58 ± 0.16 µM (P < 0.05), respectively. For oxotremorine-M-stimulated [³⁵S]GTPγS binding, the EC₅₀ values in the M₂, M₄, and M₂/M₄ wild-type groups were 0.61 ± 0.05, 0.63 ± 0.02, and 0.62 ± 0.02 µM (P < 0.05), respectively. Notably, the maximal increase in spinal [³⁵S]GTPγS binding produced by muscarine was significantly less than that stimulated by oxotremorine-M, and the EC₅₀ value of muscarine

Fig. 1. Basal [³⁵S]GTPγS binding in the spinal cord of M₂ and M₄ single-KO and M₂/M₄ double-KO mice and corresponding wild-type controls. A and B are basal-binding [³⁵S]GTPγS data in the muscarine and oxotremorine-M group, respectively. Data are presented in means ± S.E.M. (n = 4 experiments in each group).

Fig. 2. A, concentration-effect curves of [³⁵S]GTPγS binding stimulated by muscarine in the spinal cord membranes of M₂ and M₄ single-KO and M₂/M₄ double-KO mice and the three respective WT strains. B, comparison of maximal stimulated [³⁵S]GTPγS binding by 100 µM muscarine in the spinal cord from M₂ and M₄ single-KO and M₂/M₄ double-KO mice and corresponding wild-type controls. The binding data are expressed as the percentage of basal [³⁵S]GTPγS binding. Data are presented in means ± S.E.M. (n = 4). *, P < 0.05 compared with the value in the respective wild-type controls.
immunoprecipitated M2 receptors in the spinal cord between However, there was no significant difference in the amount of oxotremorine-M failed to cause any increase in \([^{35}S]\)GTP

B, attenuation of the maximal stimulated \([^{35}S]\)GTP

In M2/M4 double-KO mice, the \([^{35}S]\)GTP\(_S\) binding stimulated by muscarine and oxotremorine-M was completely eliminated (Figs. 2 and 3). By comparison, there was a small but significant reduction in stimulated \([^{35}S]\)GTP\(_S\) binding in the spinal cord of M2 single-KO mice, compared with the corresponding wild-type controls (Figs. 2 and 3). The level of attenuation of the maximal stimulated \([^{35}S]\)GTP\(_S\) binding in M4 single-KO mice was similar in muscarine- and oxotremorine-M-treated groups (14.7 \(\pm\) 0.5 versus 15.1 \(\pm\) 0.4\%). Similar to M2/M4 double-KO mice, both muscarine and oxotremorine-M failed to cause any increase in \([^{35}S]\)GTP\(_S\) binding in the spinal cord of M2 single-KO mice (Figs. 2 and 3).

Quantification of Spinal M2 Receptors in Wild-Type and mACHR KO Mice. To determine M2 receptor expression levels in the spinal cord of wild-type and mACHR KO mice (n = 4 in each group), mouse spinal cord membranes were labeled with a saturating concentration (2 nM) of the nonselective mACHR antagonist \([^{3}H]QNB\), solubilized with 1% digitonin, and then immunoprecipitated using an M2 receptor-selective antiserum (Gomeza et al., 1999a,b). This analysis showed that the three wild-type mouse strains expressed similar levels of spinal M2 receptors (data not shown). Strikingly, \([^{3}H]QNB\) binding was completely eliminated in spinal cord membranes from M2 KO mice (Fig. 4). However, there was no significant difference in the amount of immunoprecipitated M2 receptors in the spinal cord between wild-type and M4 KO mice (Fig. 4).

was about 6-fold greater than that of oxotremorine-M (Figs. 2 and 3).

DAMGO-Stimulated \([^{35}S]\)GTP\(_S\) Binding in the Spinal Cord of Wild-Type and mACHR KO Mice. To exclude the possibility that the reduction in mACHR agonist-induced \([^{35}S]\)GTP\(_S\) binding in the M4 single-KO mice was due to reduced levels of spinal cord G\(_i/o\) proteins, we carried out a series of control experiments using the \(\mu\) opioid DAMGO as an agonist. DAMGO produced a concentration-dependent increase in the \([^{35}S]\)GTP\(_S\) binding in the spinal cord of M2 and M4 KO and wild-type mice (n = 4 in each group; Fig. 5). The spinal \([^{35}S]\)GTP\(_S\) binding stimulated by DAMGO was similar among the three different wild-type strains (data not shown). Furthermore, the DAMGO-stimulated \([^{35}S]\)GTP\(_S\) binding in the spinal cord of M2 or M4 KO mice was not significantly different from that in wild-type mice (Fig. 5).

Discussion

The spinal mAChRs are important for regulation of nociception. There are five subtypes of closely homologous mAChRs that are coupled to heterotrimeric G proteins, resulting in a multitude of cellular effects through a variety of signaling pathways (Caulfield, 1993; Wess, 1996). Most available mAChR agonists or antagonists are unable to clearly discriminate among individual mAChR subtypes because of the close homology of the orthosteric binding site. Data from mACHR subtype KO mice have unequivocally demonstrated that both the M2 and M4 subtypes are involved in mediating analgesia produced by muscarinic agonists (Gomeza et al., 1999a,b; Duttaroy et al., 2002). The M2 and M4 subtypes have been demonstrated in the superficial dorsal horn of the spinal cord in rodents and humans (Pottie et al., 1996; Hoglund and Baghdoyan, 1997; Duttaroy et al., 2002;
Li et al., 2002). Also, spinal [35S]GTPγS binding stimulated by a mAChR agonist is primarily located in the superficial dorsal horn (Maher et al., 2001). In the present study, we determined the relative contribution of the M2 and M4 subtypes to activation of G1101 proteins in the spinal cord using M2 and M4 single- and M2/M4 double-KO mice.

Unlike conventional receptor binding techniques, [35S]GTPγS binding assays directly assess the efficiency of receptor-G protein coupling. It has been shown that the activation of receptors coupled to the G1101 family of G proteins is readily detected with [35S]GTPγS binding techniques (Maher et al., 2001; Sim-Selley and Childers, 2002). Thus, the M2 and M4 subtypes likely are the mAChRs measured in the present study since they are both coupled to G1101 proteins. The recently developed mAChR subtype KO mice are particularly useful experimental tools because inactivation of one specific subtype does not seem to affect the expression levels of the remaining mAChR subtypes (Wess, 2004). We found that basal [35S]GTPγS binding in the spinal cord was similar in wild-type and M2 and M4 KO mice, suggesting that basal levels of G1101 proteins and the affinity of G proteins for GDP and GTP are not altered in M2 and M4 KO mice. We observed that both muscarinic and oxotremorine-M produced profound increases in the [35S]GTPγS binding in the spinal cord of wild-type mice, indicating the presence of functional M2 and/or M4 mAChR subtypes in the spinal cord. Interestingly, oxotremorine-M displayed significantly greater potency and efficacy than muscarine in stimulating [35S]GTPγS binding in the mouse spinal cord. The level of stimulation of [35S]GTPγS binding is directly proportional to the magnitude of biological effects displayed by these two mAChR agonists in other pharmacological assays (Kurihara et al., 1993; Duttaroy et al., 2002; Chen and Pan, 2004). Thus, these data reinforce the concept that agonist-stimulated [35S]GTPγS binding is a valuable functional assay that can be used to estimate the efficacy and potency of the biological action of G protein-coupled receptor agonists.

In the spinal cord, muscarinic agonist-stimulated [35S]GTPγS binding was completely eliminated in M2/M4 double-KO mice. This observation is consistent with our recent finding that the inhibitory effect of muscarinic agonists on spinal dorsal horn neurons is abolished in rats pretreated with intrathecal pertussis toxin (Chen and Pan, 2004). Furthermore, both muscarinic and oxotremorine-M failed to increase [35S]GTPγS binding in the spinal cord of M2 single-KO mice. Radioligand binding studies suggest that the M2 subtype represents ~90% of the total spinal cord mAChR population in mice (Duttaroy et al., 2002). The [35S]GTPγS binding data therefore provide further evidence that the M2 subtype is the predominant mAChR coupled to G1101 proteins in the spinal cord.

The presence of spinal cord M4 mAChRs has been predicted based on competition radioligand binding using rat spinal cord homogenates (Hoglund and Baghdoyan, 1997). Also, because intrathecal injection of the selective M4 receptor antagonist muscarinic toxin-3 reduces the antinociceptive effect of mAChR agonists in mice (Ellis et al., 1999), functional M4 mAChRs are likely to exist in the mouse spinal cord dorsal horn. Additionally, the analgesic action of intrathecal muscarinic agonists is largely attenuated (by ~60–90%), but not abolished, in M4 single-KO mice (Duttaroy et al., 2002). Because the analgesic effect of intrathecal muscarinic agonists is completely eliminated in M2/M4 double-KO mice (Duttaroy et al., 2002), spinal M4 mAChRs are considered responsible for the residual muscarinic analgesic effect in M2 single-KO mice. However, spinal M4 mAChRs could not be detected by using immunocytochemical techniques in mice (Duttaroy et al., 2002). One possibility is that the number of M4 mAChRs in the mouse spinal cord is too low to be detected by immunocytochemical techniques. In the present study, by comparing agonist-stimulated [35S]GTPγS binding in M2 single-KO and M2/M4 double-KO and wild-type mice, we found that the M4 subtype mediated a small but significant [35S]GTPγS response in the mouse spinal cord. The [35S]GTPγS assay therefore seems to be more sensitive than the immunocytochemistry method in detecting spinal M4 mAChRs. The reduction in muscarinic agonist-stimulated spinal [35S]GTPγS binding in M4 KO mice is not due to a decrease in M4 mAChR levels because the amount of spinal M4 receptors measured by a [3H]QNB binding/immunoprecipitation assay was not significantly different between the wild-type and M4 KO mice. Also, we found that DAMGO-stimulated [35S]GTPγS binding was similar in wild-type and M2 and M4 KO mice, suggesting that the G protein levels and receptor-G protein coupling are not generally altered in mAChR KO mice. Therefore, this study provides complementary new information for the presence of a small population of functionally significant M4 mAChRs in the mouse spinal cord.

The complete loss of muscarinic agonist-stimulated [35S]GTPγS binding in M2 single-KO mice is an unexpected but important finding. Because the mAChR agonist-stimulated [35S]GTPγS binding in the spinal cord was abolished in M2/M4 double-KO mice and reduced by about 15% in M2 single-KO mice, one would predict a certain degree of residual [35S]GTPγS binding in M2 single-KO mice. Surprisingly, however, the muscarinic agonist-stimulated [35S]GTPγS binding was abolished in the spinal cord of M2 single-KO mice. These data suggest that M2 receptor activity requires the presence of M2 mAChRs in the mouse spinal cord. Alternatively, M4 mAChRs may be able to form functional oligomers with spinal M2 mAChRs. Consistent with this notion, it has been demonstrated that most G protein-coupled receptors can exist structurally or functionally as dimers or as part of larger oligomeric complexes (Angers et al., 2002; George et al., 2002).

In summary, [35S]GTPγS binding assays showed that the mouse spinal cord expresses a small but significant number of functional M4 mAChRs, in addition to the predominant population of M2 mAChRs. Importantly, our data suggest that functional activity of spinal M4 mAChRs may be dependent on the presence of M2 mAChRs, possibly reflecting the existence of functional M2/M4 mAChR oligomers. Data from this study are important for our understanding of the cellular function and drug action mediated by M2 and M4 mAChR subtypes.

References


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