Pharmacological Assessment of M₁ Muscarinic Acetylcholine Receptor-Gq/11 Protein Coupling in Membranes Prepared from Postmortem Human Brain Tissue


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ABSTRACT

Using a selective Gαq/11 protein antibody capture guanosine 5′-O-(3-[35S]thio)triphosphate ([35S]GTPS) binding approach, it has been possible to perform a quantitative pharmacological examination of the functional activity of the M₁ muscarinic acetylcholine receptor (mAChR) in membranes prepared from human postmortem cerebral cortex. Oxotremorine-M caused a 2-fold increase in [35S]GTPS-Gαq/11 binding with a pEC₅₀ of 6.06 ± 0.16 in Brodmann’s areas 23 and 25 that was almost completely inhibited by preincubation of membranes with the M₁ mAChR subtype-selective antagonist muscarinic toxin-7. In addition, the orthosteric and allosteric agonists, xanomeline [3(3-hexyloxy-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methyl[pyridine] and AC-42 (4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-buty1]-piperidine hydrogen chloride), increased [35S]GTPS-Gαq/11 binding, but with reduced intrinsic activities, inducing maximal responses that were 42 ± 1 and 44 ± 2% of the oxotremorine-M-induced response, respectively. These data indicate that the M₁ receptor is the predominant mAChR subtype coupling to the Gαq/11 G protein in these brain regions and that it is possible to quantify the potency and intrinsic activity of full and partial M₁ mAChR receptor agonists in postmortem human brain using a selective Gαq/11 protein antibody capture [35S]GTPS binding assay.

Muscarinic acetylcholine receptors (mAChRs) are widely expressed throughout the CNS. Five mAChR subtypes have been cloned (M₁, M₂, M₃, M₄, and M₅) that can be subdivided into two distinct classes based on sequence homologies and signal transduction properties. The M₁ mAChR (together with the M₅ receptor subtypes) preferentially couples to Gαq/11 proteins and stimulation of the inositol 1,4,5-trisphosphate/Ca²⁺/diacylglycerol/protein kinase C signal transduction cascade (Caulfield and Birdsall, 1998). This mAChR subtype also activates the extracellular signal-regulated kinase pathway (Berkeley et al., 2001) and modulates the activity of a diverse group of ion channels, including N-methyl-D-aspartate receptors and the M-current (Marino et al., 1998; Delmas and Brown, 2005). In contrast, M₂ and M₄ mAChRs signal through Gαo proteins to inhibit adenylyl cyclase activity and modulate a variety of other ion channels, e.g., N-type voltage-gated Ca²⁺ channels (Caulfield and Birdsall, 1998).

A variety of experimental approaches, including the use of knockout mice (Hamilton et al., 1997; Anagnostaras et al., 2003; Wess, 2004), brain lesioning, and pharmacological blockade (see Bartus, 2000), have implicated M₁ mAChR signaling in learning and memory processes (Levey, 1996; Hasselmo, 2006), and, as such, it is thought that selective activation of the M₁ mAChR subtype will provide an efficacious treatment for a variety of neurological and psychiatric disorders, including Alzheimer’s disease (Levey, 1996; Terry and Buccafusco, 2003) and schizophrenia (Raedler et al., 2007), in which cognitive dysfunction is a prominent feature of the disease.

As the potency and intrinsic activity of agonists at specific receptor subtypes can differ dramatically between experimental systems (recombinant versus native tissue) because of differences in, for example, receptor reserve (Watson et al., 2000; Nelson and Challiss, 2007), it is extremely important to

ABBREVIATIONS: mAChR, muscarinic acetylcholine receptor; CNS, central nervous system; GTPS, guanosine 5′-O-(3-thiotriphosphate); SPA, scintillation proximity assay; AC-42, 4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-buty1]-piperidine hydrogen chloride; xanomeline, 3(3-hexyloxy-1,2,5-thiadiazol-4-yl)-1,2,5,6-tetrahydro-1-methyl[pyridine]; MT-7, muscarinic toxin-7; NEM, N-ethylmaleimide.
be able to assess the pharmacological activity of putative drugs in regions of the human brain in which they are hypothesized to alter neuronal function to treat disease. To date, the majority of studies that have examined mACHR function in human brain tissue have relied on the following: 1) antagonist radioligand binding studies where agonist displacement curves have been performed in the absence and presence of GTP (Ladner et al., 1995); 2) assessment of \(^{[35]}\text{S}\text{GTP}\gamma\text{S}\) binding as an index of total G protein activation (González-Maeso et al., 2000; Scarr et al., 2006); or 3) non-membrane-based assays of more distal signaling events (Garro et al., 2001).

We have sought to improve on these approaches by developing a more specific and direct measurement of \(M_1\) mACHR function. In this respect, G protein-coupled receptor function is most directly assessed by measuring the ability of the receptor to facilitate G protein \(^{[35]}\text{S}\text{GTP}\gamma\text{S}\) for GDP exchange (Hilf et al., 1989). By combining \(^{[35]}\text{S}\text{GTP}\gamma\text{S}\) binding with an immunocapture step in which Go subtype-selective antibodies are used to recover specific Go isoform-\(^{[35]}\text{S}\text{GTP}\gamma\text{S}\) complexes, it is possible to enrich the response for specific receptor subtypes (Milligan, 2003). In this respect, DeLapp et al. (1999) have described a method that incorporates \(^{[35]}\text{S}\text{GTP}\gamma\text{S}\) binding coupled with immunocapture using Go\(_{q,11}\) antibodies and anti-IgG scintillation proximity assay (SPA) beads, which provides sufficient signal/noise to study mACHR-G\(_{q,11}\) protein coupling not only in human embryonic kidney cells expressing recombinant receptors but also in rat striatal membranes, a method that has subsequently been exploited by Porter et al. (2002) to demonstrate that oxtremoreline-M stimulates Go\(_{q,11}\)-\(^{[35]}\text{S}\text{GTP}\gamma\text{S}\) binding via activation of the \(M_1\) mACHR subtype in mouse cortex and hippocampus. We now report how this method has been modified to investigate \(M_1\) mACHR-stimulated Go\(_{q,11,\alpha}\)-GDP/GTP exchange in postmortem human cerebral cortex.

Materials and Methods

Materials. \(^{[35]}\text{S}\text{GTP}\gamma\text{S}\) (1000–1200 Ci/mmol), anti-rabbit-IgG-coated SPA beads (RPNQ0016), and protein-A-Sepharose CL-4B were obtained from GE Healthcare (Chalfont St. Giles, UK). Complete protease inhibitor cocktail was purchased from Roche Applied Science (Burgess Hill, UK). All other chemicals and reagents were obtained from Sigma-Aldrich (Poole, UK). Sprague-Dawley rats were purchased from Charles River UK Ltd. (Margate, UK). The G\(_{q,11,\alpha}\) antiserum was generated [against the C-terminal sequence (C)\(\text{LQLNLKEYNLV}\)] as described previously (Akam et al., 2001). AC-42 (Spalding et al., 2002) and xanomeline (Shannon et al., 1994) were synthesized by GlaxoSmithKline (Harlow, UK). Muscarinic toxin-7 (MT-7) was purchased from Peptides International Inc. (Louisville, KY) and prepared for use according to the manufacturer’s instructions.

Human Tissue. Normal control brain samples (Brodmann’s areas 23 and 25) from two tissue donors were obtained from the NeuroResource tissue bank at the Institute of Neurology (University College London, UK) with the appropriate ethical consent. Death-to-snap freezing time for donor 1 was 16 h. This individual was female, 68 years old, and died from a colorectal metastatic tumor. Death-to-snap-freezing time for donor 2 was 26 h. This individual was also female, 93 years old, and died from a chest infection. Neither donor displayed clinical or neuropathological evidence of neurological disease.

Membrane Preparation. Human cerebrocortical tissue from each individual donor or rat frontal cortex was homogenized using a Polytron in 10 volumes of 10 mM HEPES, pH 7.4, containing 1 mM EGTA, 1 mM dithiothreitol, 10% sucrose, and complete protease inhibitor cocktail. The resultant homogenate was diluted 10-fold and centrifuged at 1000g for 10 min at 4°C, the supernatant was saved, and the pellet was rehomogenized and centrifuged as above. The combined supernatants were then centrifuged at 11,000g for 20 min at 4°C. The resulting pellet was resuspended in buffer A at a protein concentration of 1 mg/ml, and aliquots were snap-frozen in liquid nitrogen and stored at –80°C.

N-Ethylmaleimide and Pertussis Toxin Pretreatments. A frozen aliquot of rat or human cortical membranes was diluted to the appropriate protein concentration in assay buffer containing N-ethylmaleimide (up to 10 mM; final concentration) and incubated on ice for 60 min before running the assay. Pertussis toxin pretreatment of membranes was performed as described by Hudson and Johnson (1980).

\(^{[35]}\text{S}\text{GTP}\gamma\text{S}\) Binding/Immunoprecipitation Assay. \(^{[35]}\text{S}\text{GTP}\gamma\text{S}\)-G\(_{q,11,\alpha}\) immunospecific binding as a measure of \(M_1\) mACHR activation was determined using a method modified from that described by Akam et al. (2001). In brief, frozen membrane aliquots were diluted in assay buffer (10 mM HEPES, 100 mM NaCl, 10 mM MgCl\(_2\), pH 7.4) to generate a final protein concentration of 75 \(\mu\)g of protein per 50 \(\mu\)l, which was preincubated with 0.1 \(\mu\)M GDP for 5 min on ice. Membranes (75 \(\mu\)g) were then added to buffer (40 \(\mu\)l) containing agonists and/or antagonists for 20 min at room temperature, after which 10 \(\mu\)l of \(^{[35]}\text{S}\text{GTP}\gamma\text{S}\) was added (to generate an approximate 1 nM final concentration), and the mixture was incubated for a further 5 min at 30°C. The experiment was then terminated by the addition of 1 ml of ice-cold assay buffer and immediate transfer to an ice bath. Cell membranes were recovered from the reaction mixture by centrifugation (20,000g, 6 min, 4°C). Membrane pellets were then solubilized by the addition of 50 \(\mu\)l of ice-cold solubilization buffer comprised of 100 mM Tris/HC1, 200 mM NaCl, 1 mM EDTA, and 1.25% Igepal CA-630, pH 7.4, containing 0.2% SDS. Once the pellet had been completely solubilized by vortex mixing, an equal volume of solubilization buffer without SDS was added to each tube. Solubilized protein was preclarified with normal rabbit serum (1:100 dilution) and 30 \(\mu\)l of protein A-Sepharose bead suspension [3% (w/v) in 10 mM Tris/HCl, 10 mM EDTA, pH 8.0] for 60 min at 4°C. The protein A-Sepharose beads and any insoluble material were collected in a fresh Eppendorf tube containing Go protein antiserum (1:100 dilution). Samples were vortex-mixed and rotated for 60 min at 4°C. Seventy microliters of protein A-Sepharose bead suspension was added to each sample tube, and the samples were vortex-mixed and rotated for 90 min at 4°C. Protein A-Sepharose beads were then pelleted (20,000g, 6 min, 4°C), and the supernatant was removed by aspiration. The beads were washed three times with 500 \(\mu\)l of solubilization buffer (minus SDS), and after the final wash, the recovered beads were mixed with scintillation cocktail and counted. Nonspecific binding was determined in the presence of 10 \(\mu\)M GTP\(_\gamma\text{S}\).

\(^{[35]}\text{S}\text{GTP}\gamma\text{S}\) Binding/Immunoprecipitation Assay. \(^{[35]}\text{S}\text{GTP}\gamma\text{S}\)-G\(_{q,11,\alpha}\) binding using a 96-well SPA-based method was performed using a method modified from DeLapp et al. (1999). Frozen membranes were diluted in immunoprecipitation assay buffer (20 mM HEPES, 100 mM NaCl, 10 mM MgCl\(_2\), pH 7.4) to generate a protein concentration of 25 \(\mu\)g/60 \(\mu\)l and incubated on ice for 5 min with 0.1 \(\mu\)M GDP before addition to the assay reaction. Experimental reactions were performed in a final volume of 100 \(\mu\)l in 96-well Costar plates. Buffer (20 \(\mu\)l) containing the selected agonist and/or antagonist concentrations were added to each well followed by membranes (60 \(\mu\)l), and the resultant mixture was incubated at 25°C for 20 min. After this time, \(^{[35]}\text{S}\text{GTP}\gamma\text{S}\) (20 \(\mu\)l; 0.5 mM final concentration) was added to each well, and incubations continued for a further 60 min at 25°C. The reaction was terminated by the addition of 20 \(\mu\)l of ice-cold 0.27% Igepal CA-630, and membranes were allowed to solubilize on ice for
30 min. For immunocapture of \(^{35}\text{S}\)GTP\(_S\)-Ga proteins, 10 \(\mu\)l of G\(_{\alpha1}\) antiserum was added to each well to generate a final antibody dilution of 1:300, and plates were incubated at 4°C for 60 min with shaking. Anti-IgG-coated PVT-SPA beads (50 \(\mu\)1) were then added to each well, and plates were shaken for an additional 30 min at 4°C. Plates were then centrifuged at 1000 \(\times\) g for 5 min and radioactivity determined using a Wallac MicroBeta counter.

\(^{3}H\)NMS Radioligand Binding. Cerebrocortical membranes were diluted in binding buffer (50 mM HEPES, 110 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl\(_2\), 1 mM MgSO\(_4\), 25 mM glucose, and 58 mM sucrose, pH 7.4) to generate a final protein concentration of 25 \(\mu\)g/ml. The membrane preparation (400 \(\mu\)l) was added to wells in a deep-well 96-well plate placed on ice. For each membrane preparation, total \(^{3}H\)NMS binding, nonspecific binding (10 \(\mu\)M atropine), and the component displaced by the selective M\(_1\) mACHR antagonist, MT-7 (100 nM; Adem and Karlsson, 1997), were determined. Preliminary experiments demonstrated that maximal blockade of the M\(_1\) mACHR or the total mACHR population at 4°C was achieved within 30 min of addition to membranes of 100 mM MT-7 or 10 \(\mu\)M atropine, respectively (data not shown). Therefore, after adding buffer/atropine/MT-7, plates were incubated for 30 min on ice before addition of a saturating concentration of \(^{3}H\)NMS (5 nM) and incubation at 37°C for 30 min. Bound and free \(^{3}H\)NMS was separated by filtration through a GF/B filter-mat (presoaked with 0.05% polyethyleneimine) using a 96-port cell harvester and rapid washing with 5 \(\times\) 1-ml volume of ice-cold binding buffer. \(^{3}H\)NMS retained on the filters was quantified by liquid scintillation counting.

Data Analysis. Concentration-response curves were fitted using sigmoidal nonlinear regression analysis with variable slope using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA). Inhibition constants (\(K_i\)) were determined from antagonist inhibition curves constructed in the presence of a fixed agonist concentration, using the equation of Cheng and Prusoff (1973). One-way analysis of variance was used to investigate whether maximal responses to different compounds or assay conditions were significantly different from one another. Where appropriate, Student’s \(t\) test was used to ascertain significance at a 95% confidence level.

Results

\\(^{35}\text{S}\)GTP\(_S\) Binding Studies in Rat Cerebral Cortex.\\ Preliminary experiments, using membranes prepared from adult rat frontal cerebral cortex, were performed to predict optimal assay conditions for subsequent postmortem human cerebral studies. In these experiments, oxotremorine-M-induced stimulation of \(^{35}\text{S}\)GTP\(_S\)-G\(_{\alpha1}\) coupling was greatest when membranes were preincubated with a low (0.1 \(\mu\)M) concentration of GDP, and agonist was added before adding \(^{35}\text{S}\)GTP\(_S\) to initiate the \(^{35}\text{S}\)GTP\(_S\)-G\(_{\alpha1}\) binding reaction (data not shown). Preincubating rat cortical membranes with different concentrations of the irreversible alkylating agent, N-ethylmaleimide (NEM; 0–10 mM), reduced basal levels of \(^{35}\text{S}\)GTP\(_S\) binding, resulting in a marked increase in the \(\alpha\)-fold change in \(^{35}\text{S}\)GTP\(_S\)-G\(_{\alpha1}\) binding stimulated by oxotremorine-M, the largest increase in signal being observed at 10 mM NEM, irrespective of whether immunoprecipitation or immunocapture methods were used to measure agonist activity (Fig. 1). In agreement with previous work by Flynn and Potter (1985) and Horváth et al. (1986) indicating that NEM treatment does not alter M\(_1\) mACHR agonist affinity, we have shown in Chinese hamster ovary-m1 membranes that NEM pretreatment (at concentrations up to and including 10 mM) did not alter M\(_1\) mACHR-oxotremorine-M affinity (data not shown). Pretreatment with the G\(_{\alpha1}\)-inactivating agent pertussis toxin also produced an improvement in signal/noise by reducing basal \(^{35}\text{S}\)GTP\(_S\)-G\(_{\alpha1}\) binding, but this effect was not as great, or as reproducible, as that produced by NEM pretreatment (data not shown). Thus, all subsequent human native tissue assays were performed in the presence of NEM.

\\(^{35}\text{S}\)GTP\(_S\) Binding Studies in Human Cortex.\\ In preliminary experiments using the immunoprecipitation assay format, NEM (10 mM) pretreatment markedly increased the degree of oxotremorine-M-induced \(^{35}\text{S}\)GTP\(_S\)-G\(_{\alpha1}\) binding in human cortex (Fig. 2). Subsequent experiments revealed that a 3- to 4-fold increase in maximal response could be achieved (–NEM, 139 ± 9%; +NEM, 491 ± 13% increase over basal; \(n = 3\) tissue preparations from two donors; \(p < 0.01\)), with no significant change in the oxotremorine-M EC\(_{50}\) value. These studies demonstrated that measurement of mACHR agonist-induced \(^{35}\text{S}\)GTP\(_S\)-G\(_{\alpha1}\) binding using the immunoprecipitation methodology gives rise to a high signal/noise ratio in human cortex. However, as previously mentioned, this format is low throughputs (and
uses comparatively large amounts of tissue; 75 μg of membrane protein per data point), and so a series of studies were performed to determine whether the higher throughput immunocapture methodology would generate a similarly robust data set with an adequate signal/noise ratio. This was proven to be the case in that oxotremorine-M increased [35S]GTPγS-G<sub>q/11</sub>α binding by 2-fold with a pEC<sub>50</sub> of 6.06 ± 0.16 (Fig. 3). The selective M<sub>1</sub> mACHR antagonist, MT-7, inhibited the oxotremorine-M-stimulated response with a pK<sub>i</sub> value (9.28 ± 0.33) comparable with its binding affinity for human M<sub>1</sub> mACHRs (Adem and Karlsson, 1997). Furthermore, 100 nM MT-7 almost completely abolished the oxotremorine-M-stimulated [35S]GTPγS-G<sub>q/11</sub>α binding (Fig. 3), suggesting that the M<sub>1</sub> mACHR subtype is principally responsible for the observed response to oxotremorine-M.

In a subsequent series of experiments, the pharmacological properties of the M<sub>1</sub> mACHR-selective orthosteric agonist, xanomeline (Shannon et al., 1994), was compared with those of the M<sub>1</sub> mACHR-selective allosteric agonist, AC-42 (Spalding et al., 2002; Langmead et al., 2006). Both compounds behaved as partial agonists compared with oxotremorine-M, with intrinsic activities of 42 ± 1 and 44 ± 2% and pEC<sub>50</sub> of 6.92 ± 0.09 and 6.46 ± 0.21, respectively (Fig. 4). Maximal responses evoked by xanomeline and AC-42 were significantly less than that induced by oxotremorine-M (p < 0.05). MT-7 (100 nM) abolished the [35S]GTPγS-G<sub>q/11</sub>α binding responses to EC<sub>50</sub> concentrations of xanomeline and AC-42 and reduced that induced by oxotremorine-M by ~90% (Fig. 5).

[3H]NMS Radioligand Binding Studies in Human Cortex. To estimate the relative proportions of M<sub>1</sub> versus M<sub>2</sub>-M<sub>5</sub> mACHR subtypes expressed in human cortical membranes, [3H]NMS binding studies were performed at a saturating concentration of [3H]NMS (5 nM) in the absence and presence of the broad-spectrum mACHR antagonist, atropine (to define M<sub>1</sub>- to M<sub>5</sub>-specific binding), or the selective M<sub>1</sub> mACHR antagonist, MT-7 (to define the M<sub>1</sub> mACHR binding component). Applying this methodology, we were able to demonstrate a total mACHR expression (B<sub>max</sub>) of 1043 ± 98 fmol/mg protein in the human cerebral tissue used in these studies, of which 36 ± 2% (372 ± 36 fmol/mg protein) was contributed by the M<sub>1</sub> mACHR subtype (Fig. 6).

Fig. 3. Effect of MT-7 on oxotremorine-M-stimulated G<sub>q/11</sub>α-[35S]GTPγS binding, using the SPA-based immunocapture assay. Data show a concentration-response curve for oxo-M, where no addition = 0% (basal, 687 ± 52 dpm) and oxotremorine-M (100 μM) = 100% (+oxo-M, 1393 ± 256 dpm). Concentration-dependent inhibition of the response to a submaximal concentration of oxotremorine-M (10 μM) by MT-7 is also shown. Data points are presented as means ± S.E.M. for n = 3 different membrane preparations from two donors, each performed in triplicate.

Fig. 4. Effect of increasing concentrations of oxotremorine-M, xanomeline, and AC-42 on G<sub>q/11</sub>α-[35S]GTPγS binding to human cerebral cortex membranes measured using the immunocapture assay. Data are represented as means ± S.E.M. for n = 3 different membrane preparations from two donors, each performed in triplicate.

Fig. 5. Inhibition of mACHR agonist-stimulated G<sub>q/11</sub>α-[35S]GTPγS binding to human cerebral cortex membranes by the M<sub>1</sub> mACHR-specific toxin, MT-7. Membranes were preincubated with MT-7 (100 nM) for 15 min before addition of xanomeline (300 nM), AC-42 (10 μM), or oxotremorine-M (10 μM) and assessment of G<sub>q/11</sub>α-[35S]GTPγS binding using the immunocapture assay. Data are presented as means ± S.E.M. for n = 3 different membrane preparations from two donors, each point performed in triplicate.

Discussion

There is a wealth of evidence to support the concept that selective activation of M<sub>1</sub> mACHRs should lead to cognitive benefit in a number of neurological and psychiatric disorders (Levey, 1996; Terry and Buccafusco, 2003; Raedler et al., 2007). Therefore, the M<sub>1</sub> mACHR subtype represents an attractive CNS drug target. The use of cell systems expressing near-homogeneous populations of recombinant receptors has greatly facilitated the identification of potential ligands for this receptor. However, it is increasingly clear that these systems may not always accurately reflect the functional pharmacology of agonists, and, as such, quantitative pharmacological evaluation in native tissue systems is required to provide real insight into how molecules progressing into clinical development probably modulate CNS activity and thereby treat both psychiatric and neurological disorders. In this respect, although studies in rodent tissue go some way to
rectifying the problems associated with recombinant systems, it is clearly more favorable to be able to study pharmacology in human brain tissue. To date, the majority of human brain tissue-based experiments have focused on expression studies employing immunohistochemistry, autoradiography, or radioligand binding assays to investigate whether receptor populations or downstream signaling components change with respect to their absolute level or subcellular location in postmortem brain tissue from clinically defined patient groups (Crook et al., 2001; López de Jesús et al., 2006; Scarr et al., 2006). Studies examining the downstream consequences of neurotransmitter receptor activation have been fewer in number and have relied, for example, on GTP shift analysis, using radiolabeled antagonist binding to construct agonist displacement curves in the absence and presence of GTP (Ladner et al., 1995), assessment of $[^{35}S]$GTPγS binding as an index of total G protein activation (González-Maeso et al., 2000), or evaluation of more distal signaling events, for example, through the addition of an exogenous substrate such as $[^{3}H]$phosphatidylinositol 4,5-bisphosphate to measure phospholipase C activity (Garro et al., 2001).

We now demonstrate that it is possible to use antibody capture-$[^{35}S]$GTPγS binding methods to study $\text{G}_{\alpha11}$ protein activation by the $M_1$ mAChR, thereby extending the postmortem human brain tissue assay armamentarium to $\text{G}_{\alpha11}$ protein-coupled receptors. This is an important development because $[^{35}S]$GTPγS-for-GDP exchange on non-$\text{G}_{\text{i/o}}$ G protein subpopulations is generally masked by the relatively higher rates of exchange occurring on $\text{G}_{\text{i/o}}$ proteins (Milligan, 2003), an effect that is particularly pronounced in brain tissue where $\text{G}_{\text{i}}$ (and particularly $\text{G}_{\text{o}}$) protein expression is very high (Sternweis and Robishaw, 1984). It is also noteworthy that these receptor-G protein subtype-specific coupling data have been achieved in preparations prepared from donor brains of elderly individuals (68 and 93 years of age) and after relatively long death-to-snap freezing delays (16 and 26 h).

A key aspect of the methodology used here has been the pretreatment (at 0°C) of membranes with NEM to uncouple receptors from $\text{G}_{\text{i/o}}$ proteins by irreversible alkylation of the G protein (Aktories and Jakobs, 1984; Flynn and Potter, 1985). This resulted in a substantial decrease in basal SPA-detected $[^{35}S]$GTPγS radioactivity, which created a large enough signal window to allow construction of concentration-response curves to investigate agonist pharmacology. Care needs to be taken when considering NEM pretreatment in studies of other G protein-coupled receptor subtypes; thus, previous studies have shown that unlike the situation for the $M_1$ mAChR, agonist-receptor interactions may be affected (Flynn and Potter, 1985; Horváth et al., 1986). Pertussis toxin pretreatment of membranes (which ADP-ribosylates $\text{G}_{\text{i/o}}$ proteins) caused a similar unmasking effect, but the need to preactivate the pertussis toxin to increase signal/noise made this a more time-consuming and, in our hands, less reproducible procedure. In theory, the specificity of the immunocapture method should be determined by the $\text{G}_{\alpha11}$ specificity of the antibody used (in this case, a previously characterized and highly selective $\text{G}_{\alpha11}$ polyclonal antibody; Akam et al., 2001; Selkirk et al., 2001); however, it is possible that basal (or agonist-stimulated) $\text{G}_{\alpha11}$-$[^{35}S]$GTPγS binding is sufficiently high to cause a significant background excitation of the SPA beads that would account for the reduction in basal signal produced by NEM pretreatment. An alternative explanation is that basal $\text{G}_{\alpha11}$-$[^{35}S]$GTPγS binding and/or $[^{35}S]$GTPγS-for-GDP exchange is sufficiently high to compete with activated $\text{G}_{\alpha11}$ for the available $[^{35}S]$GTPγS and that NEM pretreatment, by eliminating this competition, increases the availability of $[^{35}S]$GTPγS for $\text{G}_{\alpha11}$ binding. Whatever the case, we are confident that this treatment does not affect the pharmacology of $M_1$ mAChRs because of the following: 1) both NEM and pertussis toxin pretreatment did not significantly affect the concentration dependence of agonist activation of $M_1$ mAChRs (assessed using both the immunoprecipitation and immunocapture assay formats), and 2) NEM-mediated uncoupling of $M_2$ mAChRs from $\text{G}_{\text{i/o}}$ proteins in rat brain membranes has previously been shown to have no significant effect on the affinity state of the $M_1$ mAChR for carbachol or pirenzepine (Flynn and Potter, 1985).

A second important aspect of the current experimental protocol was the use of a low concentration of GDP (0.1 μM) optimally to facilitate $\text{G}_{\alpha11}$-$[^{35}S]$GTPγS binding (Offermanns et al., 1994; Akam et al., 2001) and sequential, rather than simultaneous (Akam et al., 2001; Selkirk et al., 2001), addition of agonist followed by $[^{35}S]$GTPγS, which improved both the magnitude and reproducibility of agonist-induced responses.

Having validated the assay as a method for measuring $\text{G}_{\alpha11}$ protein-coupled receptor activation, we deployed it to examine whether or not it was feasible to study $M_1$ mAChR pharmacology. First, because multiple mAChR subtypes are expressed in the cerebral cortex, we used the selective $M_1$ receptor toxin MT-7 (Adem and Karlsson, 1997) to establish that the vast majority (>90%) of oxotremorine-M-stimulated $\text{G}_{\alpha11}$-$[^{35}S]$GTPγS binding could be attributed to $M_1$ mAChR activation. In contrast, $[^{3}H]$NMS binding established that $M_1$ mAChRs constitute <40% of the total mAChR population. Second, we established that this assay is sufficiently sensitive to assess accurately the intrinsic activity of orthosteric site partial agonists. In this respect, xanomeline (Shannon et al., 1994), which has been reported to be an $M_1$ mAChR partial agonist in recombinant systems (Wood et al., 1999) and a functional $M_1$ mAChR antagonist in rat native tissues, presumably due to their low receptor reserve (Watson et al., 1999), behaved as a partial agonist, producing ~40% of the maximal response to...
oxtremorine-M. Third, we established that the assay could measure the activity of allosteric site agonists in that AC-42 (Spalding et al., 2002; Langmead et al., 2006) was able to stimulate G\textsubscript{M1}\textsubscript{a11} \textsuperscript{[35S]}GTP\textsubscript{S} binding to a level that at its maximum was \(~40\)% of the maximal oxtremorine-M-induced response.

These data are the first to demonstrate that the \textsuperscript{[35S]}GTP\textsubscript{S} binding/immunocapture methodology can be used to measure the potency and efficacy of both orthosteric and allosteric agonists at the M\textsubscript{3} mAChR in postmortem human brain tissue. This method may also be generally applicable to pharmacological studies of multiple G\textsubscript{M1}\textsubscript{a11} -coupled receptors expressed in native tissues and will allow comparisons to be made regarding receptor coupling in human tissue obtained from patient groups exhibiting different pathophysiologies.

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References


