M2 Muscarinic Autoreceptors Modulate Acetylcholine Release in Prefrontal Cortex of C57BL/6J Mouse

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ABSTRACT

Muscarinic autoreceptors modulate cholinergic neurotransmission in animals ranging from insects to humans. No previous studies have characterized autoreceptor modulation of acetylcholine (ACh) release in prefrontal cortex of intact mouse. Data obtained from experiments in 45 mice considered ACh as a phenotype and tested the hypothesis that pharmacologically defined M2 receptors modulate ACh release in prefrontal cortex of C57BL/6J mouse. In vivo microdialysis quantified ACh release during delivery of Ringer’s (control) or Ringer’s containing muscarinic receptor antagonists. The lowest concentration of each antagonist [scopolamine, pirenzepine, or 11-2H-pyrido(2,3-b)-1H-1-piperidinyl]-acetyl]-5,11-dihydro-6H-pyridine-one (AF-DX116)] that significantly increased ACh release was determined and defined as the minimum ACh-releasing concentration. Dialysis delivery of scopolamine caused a concentration-dependent increase in ACh release, consistent with the existence of muscarinic autoreceptors. The order of potency for causing increased ACh release was scopolamine ≈ AF-DX116 > pirenzepine. Administration of pertussis toxin into prefrontal cortex blocked the AF-DX116-induced increase in ACh release. These findings support the conclusion that M2 receptors modulate ACh release in C57BL/6J mouse prefrontal cortex. Nearly every human gene has a mouse homolog and the appeal of mouse models is reinforced by the identification of mouse genes causing phenotypic deviants. The present data encourage comparative phenotyping of cortical ACh release in additional mouse strains.

Muscarinic cholinergic autoreceptors contribute to the regulation of ACh released by both peripheral and central neurons. Muscarinic autoreceptors inhibit ACh release (Starke et al., 1989) and modulate end organ responsiveness in vascular smooth muscle of the eye (Steinle and Smith, 2000), stomach (Ogishima et al., 2000), urinary bladder (D’Agostino et al., 1997), and upper airway (Ten Berge et al., 1996a). Central cholinergic neurotransmission is altered by muscarinic autoreceptors at the level of the brain stem (Baghdoyan et al., 1998), midbrain (Kitaichi et al., 1999), forebrain (Disko et al., 1999), and cortex (Quirion et al., 1994). Muscarinic autoreceptors modulate cholinergic neurotransmission in animals ranging from insects (Judge and Leitch, 1999) to humans (Ten Berge et al., 1996b).

Disorders of cognition and memory stimulate interest in cortical autoreceptors as potential targets for drug development (Buccafusco and Terry, 2000). For example, the depletion of cortical M2 receptors in Alzheimer’s disease may reflect the loss of presynaptic autoreceptors on cholinergic terminals arising from the nucleus basalis of Meynert (Mesulam, 1998). One specific function of prefrontal cortex is manipulation of briefly stored information, or working memory (for review, see Goldman-Rakic, 1996; Gabrieli et al., 1998). Studies in rat report that ACh efflux in prefrontal cortex is enhanced during performance of a memory task (Hironaka et al., 2001). Cortical slice preparations taken from outbred strains of mice suggest that M2 autoreceptors modulate ACh outflow (Iannazzo and Majewski, 2000a, 2000b). No previous studies, however, have characterized autoreceptor modulation of ACh release from cortex of intact mouse.

Enthusiasm for murine models is reinforced by the fact that nearly every human gene has a mouse homolog (O’Brien et al., 1999). The mouse genomic sequence is anticipated to be 90% completed by 2003, enhancing genotyping and phenotyping relevant to human disease (Denny and Justice, 2000). Many mouse genes have been identified as the source of phenotypic deviants (Copeland et al., 1993; Silver, 1995) providing an important justification for the present effort to characterize central nervous system cholinergic neurotransmission as a phenotype. Chimeras crossed to the C57BL/6J

ABBREVIATIONS: ACh, acetylcholine; AF-DX116, 11-2H-pyrido(2,3-b)-1H-1-piperidinyl]-acetyl]-5,11-dihydro-6H-pyridine-one; B6, C57BL/6J; HPLC/EC, high performance liquid chromatography with electrochemical detection; mAChR, muscarinic cholinergic receptor; PTX, pertussis toxin; TTX, tetrodotoxin.
stoichiometric amounts of H2O2. A platinum electrode ionized the immobilized enzyme reaction column containing acetylcholinesterase (AChE) and choline based on molecular size and hydrophobicity. An rate

standard curve to obtain cortical levels of ACh in pmol/12.5 min. Reduced from brain samples were calculated and compared with the

then was digitized using ChromGraph software. On the day of each experiment, mice were weighed and anesthesia was induced by administering 1.5 to 2% isoflurane (Abbott Laboratories, North Chicago, IL) in 100% O2. Anesthetized mice were placed in a David Kopf (Tujunga, CA) model 962 stereotaxic frame with a model 921 mouse adapter and rat ear bars. Anesthesia was maintained by 0.70 to 0.82% isoflurane delivered through a tube covering the nose. Delivered isoflurane concentration was measured using a Raman spectrometer (Ohmeda Rascal II, Louisville, CO). Anesthetic level was evaluated constantly by observation of spontaneous movements and by response to hindlimb pinch. Respiratory rate and core body temperature were monitored every 12.5 min. Body temperature was maintained in a normal range (35.5–36.5°C) with the use of a TP400 T/Pump Heat Therapy System (Gaymar, Orchard Park, NY). The scalp was opened to expose the skull from approximately 5 mm anterior to bregma to 5 mm posterior to lambda. A small craniotomy allowed access to the brain and the microdialysis probe was aimed for prefrontal cortex, designated as frontal association cortex by the mouse brain stereotaxic atlas (Franklin and Paxinos, 1997). The aim site was 3.0 mm anterior to bregma and 1.6 mm lateral from the midline. The dorsal-ventral position of each probe was determined visually with the membrane resting fully within cortex. Probes were dialyzed continuously with Ringer’s solution.

Experimental Design. After probe insertion, several initial dialysis samples were collected to ensure stable baseline ACh release from prefrontal cortex. Six dialysis samples (75 min) then were collected and the ACh quantified from these samples was averaged to determine baseline ACh release. A CMA/110 liquid switch then was used to change the dialysate from Ringer’s alone to Ringer’s containing a drug, allowing uninterrupted dialysis. Prefrontal cortex was dialyzed with either scopolamine (1, 3, 10, 30, or 100 nM), AF-DX116 (0.3, 1, 3, or 30 nM), pirenzepine (100 or 300 nM), or TTX (1 μM) while an additional six dialysis samples (75 min) were collected. Drug-induced changes in ACh release are presented as percent change from control and are expressed as mean ± S.E.M. Only one drug concentration was tested per experiment and each mouse was used for only one experiment. At the conclusion of each experiment, the microdialysis probe was removed from the cortex, the scalp wound was closed with wound clips, and the mouse was allowed to recover overnight.

One goal of the present study was to identify the mAChR subtype modulating ACh release in mouse prefrontal cortex. The lack of subtype-selective mAChR ligands presents a challenge for studies aiming to identify functional roles of mAChR subtypes. Whereas no purely subtype-selective mAChR agonists are available, a number of antagonists display different binding affinities for the five mAChR subtypes (Caulfield and Birdsall, 1998). No single antagonist, however, has high affinity for one subtype and low affinity for the remaining four mAChRs (Caulfield and Birdsal, 1998). Fortunately, several relatively subtype-selective muscarinic antagonists can be used in concert to distinguish mAChR subtypes in functional assays. In the present study, the relative potencies of three mAChR antagonists (scopolamine, AF-DX116, and pirenzepine) for increasing cortical ACh release were compared with previously determined affinities of these antagonists for the five mAChR subtypes. Potencies were determined by finding the lowest mAChR antagonist concentration eliciting a significant increase in ACh release, defined as the minimum ACh-releasing concentration (Billard et al., 1995). The

Materials and Methods

Animal Care. Animal handling and procedures were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (National Academy of Sciences Press, 1996). Adult male B6 mice (n = 45, 25–35 g; Jackson Laboratory, Bar Harbor, ME) were housed in a 12:12 light/dark cycle with free access to food and water. All experiments took place between 10 AM and 5 PM.

Drug Preparation. Scopolamine methyl bromide, pirenzepine dihydrochloride, tetrodotoxin (TTX), and pertussis toxin (PTX) (Sigma, St. Louis, MO) were dissolved in Ringer’s solution (147 mM NaCl, 2.4 mM CaCl2, 4.0 mM KCl, and 10 mM HEPES, pH 7.4) to produce a 25 mg/ml stock. To prepare the final test solution, 25 mg/ml was diluted 1:1 with Ringer’s solution (147 mM NaCl, 2.4 mM CaCl2, 4.0 mM KCl, and 10 mM HEPES, pH 7.4). A constant rate of 2.0 μl/min with a syringe was used for each experiment. At the conclusion of each experiment, the microdialysis probe was removed from the cortex, the scalp wound was closed with wound clips, and the mouse was allowed to recover overnight.

In Vivo Microdialysis and High Performance Liquid Chromatography with Electrochemical Detection (HPLC/EC). CMA/11 microdialysis probes (CMA/Microdialysis, Acton, MA) measuring 1.0 mm in length and 0.24 mm in diameter were used for all experiments. The molecular weight cutoff of the CMA/11 cuprophane dialysis membrane is 6,000 Da. The molecular weight of ACh is less than 150 Da. IBMAT-2 combination microdialysis/microinjection probes (Bioanalytical Systems, Inc., West Lafayette, IN) measuring 1.0 mm in length and 0.36 mm in diameter with a 30,000 Da molecular weight cutoff were used for all experiments. Ringer’s and drug solutions were delivered to the dialysis probe at a constant rate of 2.0 μl/min with a syringe. Dialysis samples of 25 μl were collected every 12.5 min.

Samples collected for quantification of ACh were injected into an HPLC/EC (Bioanalytical Systems, Inc., West Lafayette, IN) system, as described previously (Baghdoyan et al., 1998; Mortazavi et al., 1999). Upon injection, a 50 mM Na3HPO4 (pH 8.5) mobile phase (flow rate = 1.0 ml/min) carried the sample to a column that separates ACh and choline based on molecular size and hydrophobicity. An immobilized enzyme reaction column containing acetylcholinesterase and choline oxidase then converted the ACh and choline to stoichiometric amounts of H2O2. A platinum electrode ionized the H2O2 with an applied potential of 500 mV in reference to a Ag/AgCl electrode. The resulting signal created a chromatographic peak that then was digitized using ChromGraph software. On the day of each experiment, five known amounts of ACh (0.2, 0.3, 0.4, 0.5, and 1.0 pmol) were injected in triplicate into the HPLC/EC system to create a standard curve. The areas under the chromatographic peaks produced from brain samples were calculated and compared with the standard curve to obtain cortical levels of ACh in pmol/12.5 min.

Before every experiment, the microdialysis probe was used to dialyze a known concentration of ACh to determine the percentage of ACh recovered by the probe. At the end of each experiment, probe recovery of ACh was again quantified by placing the dialysis probe in the same known concentration of ACh. Probe recoveries were determined before and after each experiment and were compared by t tests. The purpose of this procedure was to ensure that probe membrane properties had not changed during the course of the experiment. All experimental data reported are from experiments in which pre- and postexperimen probe recoveries were not significantly different.

Anesthesia and Animal Preparation. At the beginning of each experiment, mice were weighed and anesthesia was induced by administering 1.5 to 2% isoflurane (Abbott Laboratories, North Chicago, IL) in 100% O2. Anesthetized mice were placed in a David Kopf (Tujunga, CA) model 962 stereotaxic frame with a model 921 mouse adapter and rat ear bars. Anesthesia was maintained by 0.70 to 0.82% isoflurane delivered through a tube covering the nose. Delivered isoflurane concentration was measured using a Raman spectrometer (Ohmeda Rascal II, Louisville, CO). Anesthetic level was evaluated constantly by observation of spontaneous movements and by response to hindlimb pinch. Respiratory rate and core body temperature were monitored every 12.5 min. Body temperature was maintained in a normal range (35.5–36.5°C) with the use of a TP400 T/Pump Heat Therapy System (Gaymar, Orchard Park, NY). The scalp was opened to expose the skull from approximately 5 mm anterior to bregma to 5 mm posterior to lambda. A small craniotomy allowed access to the brain and the microdialysis probe was aimed for prefrontal cortex, designated as frontal association cortex by the mouse brain stereotaxic atlas (Franklin and Paxinos, 1997). The aim site was 3.0 mm anterior to bregma and 1.6 mm lateral from the midline. The dorsal-ventral position of each probe was determined visually with the membrane resting fully within cortex. Probes were dialyzed continuously with Ringer’s solution.

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One goal of the present study was to identify the mAChR subtype modulating ACh release in mouse prefrontal cortex. The lack of subtype-selective mAChR ligands presents a challenge for studies aiming to identify functional roles of mAChR subtypes. Whereas no purely subtype-selective mAChR agonists are available, a number of antagonists display different binding affinities for the five mAChR subtypes (Caulfield and Birdsall, 1998). No single antagonist, however, has high affinity for one subtype and low affinity for the remaining four mAChRs (Caulfield and Birdsall, 1998). Fortunately, several relatively subtype-selective muscarinic antagonists can be used in concert to distinguish mAChR subtypes in functional assays. In the present study, the relative potencies of three mAChR antagonists (scopolamine, AF-DX116, and pirenzepine) for increasing cortical ACh release were compared with previously determined affinities of these antagonists for the five mAChR subtypes. Potencies were determined by finding the lowest mAChR antagonist concentration eliciting a significant increase in ACh release, defined as the minimum ACh-releasing concentration (Billard et al., 1995). The...
maximum non-ACh-releasing concentration, defined as the highest drug concentration having no significant effect on ACh release (Billard et al., 1995), also was determined for each antagonist.

Minimum ACh-releasing concentrations and maximum non-ACh-releasing concentrations were determined according to the following procedure. An initial drug concentration was tested in vivo by microdialysis delivery. If the first concentration had no effect on ACh release, a 0.5 log unit higher concentration of the same drug was tested. This procedure of increasing the antagonist concentration by 0.5 log unit was repeated until a significant increase in ACh release was elicited. Similarly, if the first drug concentration did cause a significant increase in ACh release, then a 0.5 log unit lower concentration of the same drug was tested. This procedure was repeated until a concentration was found which elicited no change in ACh release. Minimum ACh-releasing concentrations and maximum non-ACh-releasing concentrations were determined with at least three mice for each drug.

The present study also used PTX as a tool to identify the mAChR subtype modulating ACh release in prefrontal cortex. M2/M4 mAChRs are coupled to guanine nucleotide-binding (G) proteins of the Gi/Go subtype (Caulfield and Birdsall, 1998). PTX acts to ADP-ribosylate the α-subunit and uncouple the inhibitory G protein (Carty, 1994). PTX blockade of AF-DX116-evoked ACh release would provide novel support for modulation of cortical ACh release by autoreceptors of the M2/M4 subtype. The IBR-2 dialysis probe also contained a microinjection port making it possible to administer vehicle (control) or PTX (5 ng/100 nl) into the cortical dialysis site. These combined microinjection and microdialysis experiments made it possible to compare AF-DX116-evoked ACh release after microinjection of vehicle alone to AF-DX116-evoked ACh release after microinjecting PTX.

Histology. After each experiment, brains were removed for histological processing to confirm probe placements. Brains were frozen in a bromobutane/isopentane bilayer, then mounted for coronal sectioning at 40 μm on a Bright model OTF cryostat (Huntingdon, Cambs, England). Cut sections were thaw-mounted onto gelatin coated slides, dried for 2 h under vacuum desiccation, and then fixed with paraformaldehyde vapors at 80°C. The fixed sections were stained with cresyl violet, backlit with a Northern Light illuminator (Imaging Research, St. Catherine’s, ON, Canada) and digitized with a Cohu (San Diego, CA) charge-coupled device camera connected to a Macintosh G-3 computer. These digitized images were used to localize microdialysis probe placements as compared with a stereotaxic atlas (Franklin and Paxinos, 1997). Only results from experiments in which the dialysis probes were localized fully within prefrontal cortex are reported.

Data Analysis. Fifteen mice were used for the scopolamine concentration-response study (three mice per concentration) and data were analyzed by repeated measures of one-way analysis of variance followed by a Dunnett’s test. The scopolamine, AF-DX116, and pirenzepine minimum-releasing concentration studies were conducted using six mice each and analyzed by t test. Data collected for the TTX study were obtained from three mice and analyzed by t test. Pertussis toxin studies were conducted in nine mice, and statistical significance was tested with analysis of variance, t test, and Mann-Whitney U test. Degrees of freedom and probability values are provided under Results.

Results

Histological examination verified that microdialysis probes were placed fully within the prefrontal cortex. Figure 1A shows a digitized cresyl violet-stained coronal mouse brain section. This image illustrates a typical microdialysis probe site. Stereotaxic coordinates for the center of each probe were localized along the rostral-caudal axis by comparison to the mouse brain atlas (Franklin and Paxinos, 1997). A metric ruler was digitized with each sectioned brain to calibrate the position of each dialysis probe along the medial-lateral and dorsal-ventral axes. Figure 1, B and C, shows the probe centers from 45 experiments in relation to a B6 brain viewed from above (Rosen et al., 2000). Rostral-caudal probe center placements ranged from 2.78 to 3.06 mm anterior to bregma. Medial-lateral probe center placements ranged from 1.4 to 2.1 mm from the midline. Dorsal-ventral probe center placements ranged from 0.5 to 0.8 mm below the surface of the cortex.

Figure 2 shows that microdialysis delivery of 1 μM TTX decreased ACh release in prefrontal cortex. Figure 2A illustrates a typical time course of ACh release as a function of sequential dialysis sample. Figure 2B shows data summarized from three experiments. TTX significantly decreased ACh release by 55% (t = 5.5; df = 34; p < 0.0001). These findings are consistent with the view that ACh measured in this study represents vesicular release.

The effect of scopolamine on the time course of ACh release during three separate experiments is shown in Fig. 3. Microdialysis administration of 1 nM scopolamine caused no change in ACh levels. In contrast, dialysis delivery of 10 nM
Scopolamine began to increase ACh release by the 100-min time point. Dialysis delivery of 100 nM scopolamine caused an increase in ACh release within the first 12.5 min of delivery.

Figure 4 summarizes results from experiments in 15 mice showing that dialysis delivery of scopolamine caused a concentration-dependent increase in ACh release (F = 40.5; df = 4, 72; p < 0.0001). Multiple comparisons statistic (Dunnett’s) showed that microdialysis delivery of 0 nM scopolamine caused no significant change in ACh release. Delivery of 1 nM scopolamine caused a 14 ± 3.8% increase over control levels. A concentration of 10 nM scopolamine caused a 24 ± 3.5% increase in ACh release. Higher concentrations of scopolamine delivered by microdialysis caused greater increases in ACh release: 30 nM scopolamine induced an average 87 ± 17.2% increase, whereas 100 nM scopolamine induced an average 180 ± 23.4% increase in ACh release. Asterisks indicate significant (p < 0.05) increases compared with control.

Figure 5 summarizes the results of nine experiments designed to determine the effect of PTX on AF-DX116-stimulated ACh release. Delivery of 1 nM AF-DX116 into prefrontal cortex caused a significant (p < 0.001) increase in ACh release over control levels. Scopolamine and AF-DX116 were most potent and pirenzepine was least potent in significantly increasing ACh release. This order of potency suggests that the M2 subtype functions as an autoreceptor in mouse prefrontal cortex.
these M2 receptors are autoreceptors. Postsynaptic heteroreceptors may have caused increased ACh release via feedback circuitry. Evidence from cortical slice release, however, supports locally mediated enhancement of cortical ACh release (Iannazzo and Majewski, 2000a). The decreased ACh levels caused by TTX (Fig. 2) are consistent with the likelihood that ACh measured by microdialysis reflects synaptic release, rather than changes in ACh turnover. This interpretation follows from the fact that TTX eliminates the propagation of action potentials by blocking sodium channels (Catterall, 1992).

The present ACh measures also are limited to autoreceptor modulation of ACh release during isoflurane anesthesia. The most compelling reason for choosing an anesthetized animal model is that cortical ACh levels vary significantly with the sleep-wake cycle (Jasper and Tessier, 1971; Marroussi et al., 1995). Thus, anesthesia was used to hold the arousal state constant while permitting ACh levels to fluctuate in response to antagonist administration. Used in this way, anesthesia has been shown to be a useful tool for in vivo microdialysis studies of pontine (Baghdoyan et al., 1998) and cortical (Materi et al., 2000) ACh release.

The homology of rodent frontal association cortex to primate prefrontal cortex has been questioned (Preuss, 1995). As illustrated in Fig. 1, localization of microdialysis sites was confirmed by comparison of histological sections with the mouse brain atlas (Franklin and Paxinos, 1997). Similar to primate prefrontal cortex, rodent frontal association cortex can be defined by its interconnectivity with other brain regions, specifically the mediodorsal thalamus, amygdala, striatum, and monoaminergic nuclei (Groenewegen and Uylings, 2000). These anatomical criteria suggest that mouse frontal association cortex (Franklin and Paxinos, 1997) is homologous to primate prefrontal cortex.

**Multiple Lines of Evidence for M2 Autoreceptor Sub-type in Prefrontal Cortex of B6 Mouse.** Scopolamine caused an increase in cortical ACh levels (Figs. 3 and 4). The scopolamine effects on ACh release were rapid in onset (Fig. 3), sustained for the duration of antagonist delivery (Fig. 3), and concentration-dependent (Fig. 4). These data encouraged efforts to identify the mAChR subtype modulating cortical ACh release. The Fig. 5 results show minimum ACh-releasing concentrations of 3 nM for scopolamine and AF-DX116 and 300 nM for pirenzepine.

The likelihood that the M2 subtype modulates cortical ACh release is based on the following rationale. Scopolamine has equal and high affinity for all mAChR subtypes and AF-DX116 has a higher affinity for the M2 and M4 subtypes than for the M1, M3, or M5 subtypes (Billard et al., 1995). AF-DX116 and scopolamine were equipotent for increasing ACh release, favoring the interpretation that M2 or M4 receptors are more important than M1, M3, or M5 receptors in modulating ACh release. Pirenzepine has a higher affinity for the M1 and M4 subtypes than for the M2, M3, or M5 subtypes (Caulfield and Birdsell, 1998). If the autoreceptor were of the M1 subtype, pirenzepine would be predicted to be equipotent to scopolamine and more potent than AF-DX116 for causing increased ACh release. If the autoreceptor were of the M4 subtype, pirenzepine would be expected to exhibit a minimum ACh-releasing concentration similar to that of scopolamine and AF-DX116. Instead, a concentration of pirenzepine 2 log units greater than that of scopolamine and AF-DX116 was required to significantly increase ACh release.

**Discussion**

Three main findings emerged from the present study. First, microdialysis delivery of scopolamine to prefrontal cortex caused a concentration-dependent increase in ACh release, consistent with the hypothesis that ACh release in mouse prefrontal cortex is modulated by muscarinic autoreceptors. Second, quantification of the minimum ACh-releasing concentrations for three mAChR antagonists revealed the order of potency to be scopolamine = AF-DX116 > pirenzepine. This finding suggests that the M2 subtype modulates ACh release in prefrontal cortex of C6 mouse. Third, the discovery that pertussis toxin blocked AF-DX116-evoked ACh release is consistent with M2 autoreceptors functioning as modulators of ACh release. These three findings are considered below after noting the limitations of this study.

**Limitations.** Potential methodological limitations include the presence of neostigmine in the dialysis solution (Ringer’s), diffusion of antagonists away from the microdialysis probe, and the possibility of ACh release increasing as a result of antagonist binding to postsynaptic mAChRs. These issues have been discussed in detail elsewhere (Billard et al., 1995; Baghdoyan et al., 1998). It must be acknowledged that although the present study clearly demonstrates modulation of ACh release by M2 receptors, the data do not prove that these M2 receptors are autoreceptors. Postsynaptic heteroreceptors have been discussed in detail elsewhere (Billard et al., 1995; Baghdoyan et al., 1998). It must be acknowledged that although the present study clearly demonstrates modulation of ACh release by M2 receptors, the data do not prove that these M2 receptors are autoreceptors.
The findings argue against the M1 or M4 subtypes as candidates for the autoreceptors modulating ACh release in mouse prefrontal cortex. Muscarinic autoreceptors of the M2 subtype, however, would be expected to show the order of potency observed in the present study: scopolamine = AF-DX116 > pirenzepine. Finally, pertussis toxin blocking of the AF-DX116-evoked increase in ACh release (Fig. 6) is consistent with the interpretation that AF-DX116 antagonized M2 mAChRs coupled to pertussis toxin-sensitive G proteins. Thus, although the M1 subtype is the most prevalent in rodent cortex (Levey et al., 1991), it may be inferred from these data that the B6 mouse cortical autoreceptor is likely of the M2 subtype.

There is good agreement that vertebrate presynaptic muscarinic receptors are typically metabolotropic and inhibit transmitter release (Schmitz et al., 2001). Recent in vitro studies have implicated the M2 subtype in cortical autoreceptor function in brain slice preparations from outbred strains of mice (Iannazzo and Majewski, 2000a) and from M2 receptor knockout mice (Zhang et al., 2001). Presynaptic M2 receptors interact with the vesicular release-related proteins syntaxin and SNAP-25 as a function of agonist binding and cell excitability (Linial et al., 1997; Ilouz et al., 1999). Together, these data support the concept of the M2 receptor modulating ACh release.

**Functional Implications of M2 Autoreceptors as Modulators of ACh Release in Prefrontal Cortex.** The present ACh release data from anesthetized mouse cannot directly address autonomic or behavioral functions regulated by prefrontal cortex (Uylings et al., 2000). Evidence that ACh release in prefrontal cortex is modulated by M2 receptors, however, does have clear functional implications. ACh is understood to be an essential regulator of cortical excitability (reviewed in Sarter and Bruno, 2000). The prefrontal cortex contributes to the regulation of attention and arousal (Groeneveld and Uylings, 2000). Deactivation of prefrontal cortex has been interpreted as “the single most salient feature common to both nonrapid eye movement and rapid eye movement sleep and may be a defining characteristic of sleep per se” (Braun et al., 1997). Behavioral (Horne, 1993; Harrison and Horne, 1997) and functional MRI (Drummond et al., 1999) data show that sleep deprivation interferes with consolidation of working memory. The foregoing data are consistent with the view that prefrontal cortical function is particularly vulnerable to sleep deprivation (Horne, 1993; Harrison and Horne, 1997) and anesthesia (Andrade, 1996; Casele-Rondi, 1996). Autoreceptor loss has been suggested as a factor contributing to Alzheimer’s disease (Mesulam, 1998) characterized by cognitive decline and sleep disruptions. Age-related decrements in mAChR density recently have been demonstrated in human frontal cortex by PET imaging studies (Zubieta et al., 2001). Thus, the present results are consistent with functional evidence that cell excitability in prefrontal cortex is altered by mAChRs (Haj-Dahmame and Andrade, 1998).

**Conclusions.** The conclusion that ACh release is modulated by autoreceptors of the M2 subtype may be limited to B6 mouse. The results encourage future studies aiming to determine whether the M2 subtype modulates ACh release in different mouse strains. Such comparative studies may contribute to a better understanding of autoreceptors as a protein phenotype expressed by genetically distinct strains of mice. The relative value of using ACh release as a phenotype versus other pharmacological and behavioral methods remains to be demonstrated. The present data provide an essential first step in the evaluation of ACh as a phenotype versus other measures. Elucidating presynaptic mechanisms regulating cholinergic neurotransmission may lead to pharmacological strategies that can offset disease-related reductions in cortical ACh release (Buccafusco and Tercy, 2000).

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