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Pharmacological and Ionic Characterizations of the Muscarinic Receptors Modulating [^{3}H]Acetylcholine Release from Rat Cortical Synaptosomes[1]

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Abstract

The muscarinic receptors that modulate acetylcholine release from rat cortical synaptosomes were characterized with respect to sensitivity to drugs that act selectively at M_1 or M_2 receptor subtypes, as well as to changes in ionic strength and membrane potential. The modulatory receptors appear to be of the M_2 type, since they are activated by carbachol, acetylcholine, methacholine, oxotremorine, and bethanechol, but not by pilocarpine, and are blocked by atropine, scopolamine, and gallamine (at high concentrations), but not by pirenzepine or dicyclomine. The ED_{50}s for carbachol, acetylcholine, and oxotremorine are less than 10 μM, suggesting that the high affinity state of the receptor is functional. High ionic strength induced by raising the NaCl concentration has no effect on agonist (oxotremorine) potency, but increases the efficacy of this compound, which disagrees with receptor-binding studies. On the other hand, depolarization with either KCl or with veratridine (20 μM) reduces agonist potencies by approximately an order of magnitude, suggesting a potential mechanism for receptor regulation.

Presynaptic muscarinic receptors on central and peripheral cholinergic terminals are known to attenuate acetylcholine (ACh) release when activated (Starke, 1981; Briggs and Cooper, 1982; Raiteri et al., 1984). This receptor-mediated modulation of cholinergic transmission appears important physiologically and is a potential site for pharmacological intervention as well (Starke, 1981). However, little is known about the receptor subtypes mediating release modulation or about the intracellular processes that transduce receptor activation into a change in transmitter release.

Recent studies indicate that there are at least two muscarinic receptor subtypes, classified M_1 and M_2, that differ anatomically and with respect to agonist and antagonist binding properties (Potter et al., 1984). M_1-receptors appear to be predominately localized postsynaptically throughout the brain and in several peripheral organs that are innervated cholinergically (Potter et al., 1984), and their activation results in increased phosphatidylinostiol turnover in the rat brain (Gonzales and Crews, 1984). M_2-receptors, however, appear pre- and postsynaptically in brain, are regulated by an intrinsic membrane protein that binds to GTP (g-protein), and may not be coupled to changes in phosphatidylinositol turnover.

The present studies were designed to determine whether M_1- or M_2-receptors mediate the presynaptic modulation of ACh release. These studies involve dose-response curves for the release of synaptosomal [^{3}H]ACh in the presence of selected muscarinic agonists and antagonists, as well as treatments that selectively alter M_1- or M_2-receptor activity. Our results indicate that the presynaptic modulation of [^{3}H]ACh release is mediated by M_2- but not M_1-receptors. Furthermore, we find that this M_2-receptor activation by agonists is sensitive to changes in membrane potential, which suggests a possible mechanism for release regulation.

Materials and Methods

[*methyl*-^{3}H]Choline (80 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Oxotremorine, atropine, choline kinase, methylcholine, pilocarpine, scopolamine, bethanechol, gallamine, acetylcholine chloride, guanylylimidodiphosphate (GppNHp), and hexamethonium were purchased from Sigma Chemical Co. (St. Louis, MO). Pirenzepine and dicyclomine were generous gifts from Dr. Lincoln Potter (University of Miami) and Dr. John Downie (Dalhousie University), respectively. All solutions were prepared in Krebs-Ringer (KR) solution (Meyer and Cooper, 1981).

Synaptosomal preparation. Adult male Sprague-Dawley rats (175 to 225 gm) were decapitated and their cortices were removed to prepare a crude synaptosomal P_2 fraction as described previously (Sgaragli et al., 1977). The synaptosomes were washed and then resuspended in 2.5 ml oxygenated KR buffer. To load the synaptosomes with [^{3}H]ACh, they were incubated with 1 μM [^{3}H]choline for 10 min at 37°C. They were then washed and resuspended in up to 20 ml of KR containing 50 μM eserine. Aliquots of the synaptosomal suspension (0.3 ml, containing approximately 0.4 mg of protein) were added to 0.7 ml of the same buffer, preincubated for 30 sec at 37°C, with or without specified cholinergic agents. A depolarizing concentration of KCl or an equivalent amount of NaCl was then added to some tubes and the incubation was terminated 2 min later by cooling on ice for 4 min, followed by centrifugation at 12,000 × *g* for 4 min. Controls for release were left on ice-cold KR. It should be noted that muscarinic release modulation was not observed consistently at higher synaptosomal protein concentrations, perhaps due to the presence of endogenous modulatory substances.

[^{3}H]ACh and [^{3}H]choline determinations. [^{3}H]ACh was separated from choline in the synaptosomal supernatants by liquid cation exchange as described previously (Nemeth and Cooper, 1978) and modified as follows: 0.011 ml of 0.1 N HCl was added to 0.1-ml aliquots of the supernatant. Glyclyglycine buffer (0.1 ml of 0.4 M glycylglycine, 20 mM $MgCl_2$, and 20 mM sodium ATP) and 0.0012 unit of choline kinase (1.2 units/ml) were added. After incubating for 45 min at 37°C, the reaction was stopped by adding 0.4 ml of H_2O. Sodium tetraphenylboron (10 mg) in 1 ml of butyronitrile was added and the tubes were thoroughly vortexed. The aqueous and organic phases were separated by centrifugation at 1000 × *g* for 5 min. The organic fraction containing [^{3}H]ACh and the aqueous fraction containing labeled

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choline were counted in Liquiscint (National Diagnostics, Somerville, NJ), by a Beckman LS 1800 scintillation counter.

Protein determinations. Synaptosomal protein levels were determined using the Bio-Rad procedure involving Coomassie blue staining of peptides.

Statistical analyses. Comparisons among multiple means were performed with the q test (Dixon and Massey, 1969).

Results

The spontaneous, temperature-dependent efflux of labeled ACh was observed during a short, 2.5-min incubation at 37°C that was found in preliminary experiments to optimize muscarinic release modulation, when expressed as percentage of control values. Percentage of control values for each cortical P_2 preparation were determined from the formula: % control value = (dpm of [^{3}H]ACh released in presence of treatment/mg of protein in sample) ÷ (dpm of [^{3}H]ACh released in absence of treatment/mg of protein). An identical calculation was performed to determine the percentage of [^{3}H]choline released as well. The release modulation under these spontaneous release conditions is calcium dependent and apparently mediated by changes in voltage-dependent calcium uptake (E. St. Onge and E. Meyer, manuscript submitted for publication).

Various muscarinic agonists inhibited [^{3}H]ACh release in a dose-dependent manner (Fig. 1), and [^{3}H]choline efflux was not modulated by any of these agents (not shown), indicating a modulation of the calcium-dependent release process as we previously reported. Oxotremorine and acetylcholine are the most potent release modulators, with EC_{50}s of about 5 μM. Carbachol (EC_{50} about 5 μM) was slightly less potent, and bethanechol (EC_{50} about 100 μM) was much less potent, although all of these agonists had similar efficacies with respect to maximal release inhibition. In contrast, pilocarpine had no effect on [^{3}H]ACh release, even at very high concentrations. Interestingly, acetylcholine and methacholine appeared to have complicated dose-related effects on transmitter release and, at high concentrations, actually inhibited [^{3}H]ACh release less than at intermediate concentrations. This reduced modulation was not always observed at high concentrations of ACh or methacholine, but was blocked with 500 μM hexamethonium when it did occur (not shown), suggesting a nicotinic stimulation of transmitter release.

Further evidence that M_2 muscarinic receptors mediate release modulation in this preparation derives from the antagonist dose-response curves shown in Figure 2. Atropine and scopolamine each blocked oxotremorine-induced inhibition of [^{3}H]ACh release with similar potency (ED_{50}s about 1 μM), whereas pirenzepine had no significant effect on this release modulation. Gallamine, which has been shown to allosterically modify M_2- and M_1-receptors in binding studies (Potter et al., 1984), had little effect on transmitter release until a high concentration (100 μM) was reached. The reason that such a high concentration was required is not clear, and may suggest that the drug's allosteric action alone has little effect on ACh release. Dicyclomine, which appears to modify M_1 but not M_2 receptors, had no effect on release modulation even at high concentrations. Hexamethonium also had no effect on oxotremorine-induced transmitter release modulation.

The potencies of oxotremorine, ACh, carbachol, bethanechol, and methacholine with respect to inhibiting [^{3}H]ACh release appeared to decrease by severalfold in the presence of a depolarizing, 30 mM concentration of K^+ (Fig. 3), when compared to normal KR buffer. For each of these drugs, however, the efficacies were only slightly altered by K^+ when expressed as percentage of control values. Pilocarpine again had no effect on transmitter release in the presence of this depolarizing K^+ concentration.

In order to characterize further the effect of K^+ depolarization on muscarinic inhibition of transmitter release, we determined the effects of different concentrations of this ion or Na^+ on [^{3}H]ACh release in the presence or absence of 100 μM oxotremorine (Figs. 4 and 5). The difference between the [^{3}H]ACh released in the presence or absence of this agonist was defined as the oxotremorine-induced inhibition of release. The oxotremorine-induced inhibition of [^{3}H]ACh release depended on K^+ concentration, with less inhibition seen at high K^+ concentrations (q value for each K^+ concentration over 5.5 mM K^+ was at least 12.50, $p < 0.01$, compared to control value). Interestingly, replacement of the K^+ with equal concentrations of Na^+ did not reduce oxotremorine-induced modulation and actually increased modulation at high levels of osmolarity. Thus, the K^+-dependent reduction in oxotremorine-induced modulation of [^{3}H]ACh release was not due to changes in osmotic strength alone.

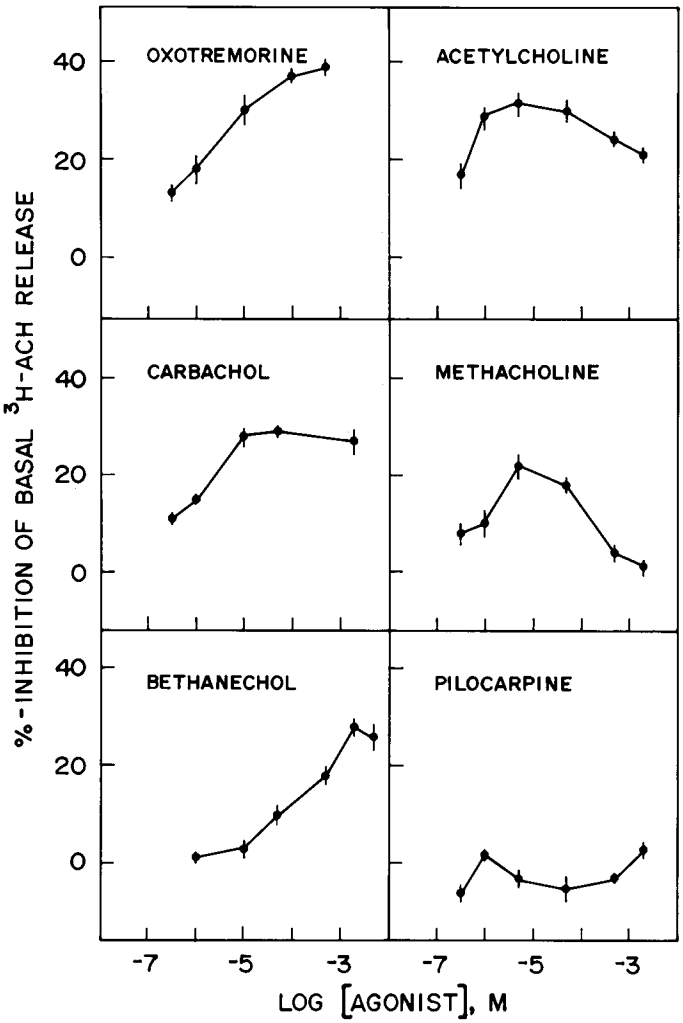

Figure 1. Dose-response curves for muscarinic agonist-induced inhibition of [^{3}H]ACh release. Rat cortical synaptosomes that were preloaded with [^{3}H]ACh were incubated with the specified concentration of muscarinic agonist for 2.5 min at 37°C, and the [^{3}H]ACh released into the medium was assayed and expressed as the percentage of control value ± SEM (N = 4 to 5 animals/group). The percentage inhibition of release was determined by subtracting the percentage of control value for each treatment from 100%.

Figure 6 shows that the dose-response curve for [^{3}H]ACh release versus oxotremorine concentration also was affected differentially by K^+ and Na^+ ions. Whereas hyperosmolarity induced with 80 mM Na^+ appeared to increase the efficacy and had no effect on the potency of oxotremorine-induced [^{3}H]ACh release attenuation, 80 mM K^+ clearly reduced the potency of this muscarinic agonist, as denoted by the right-shift of the curve. The concentration of oxotremorine necessary to inhibit ACh release maximally clearly increased with elevated K^+ (cf. Figs. 1, 4, and 6).

In order to determine whether depolarization was sufficient to reduce agonist potency, synaptosomes were treated with a depolarizing concentration (20 μM) of veratridine and increasing concentrations of oxotremorine (Fig. 7). The oxotremorine dose-response

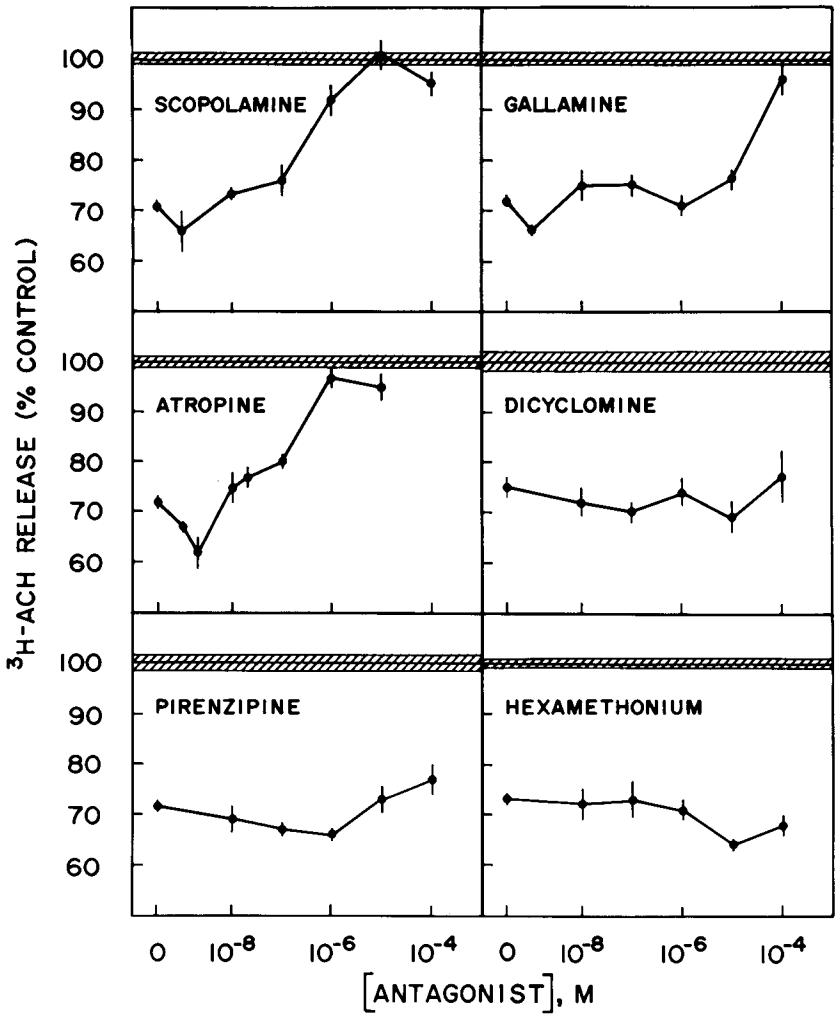

Figure 2. Dose-response curves for muscarinic antagonist-induced blockade of the oxotremorine-induced inhibition of [^{3}H]ACh release. Rat cortical synaptosomes that were preloaded with [^{3}H]ACh were incubated with the specified concentration of muscarinic antagonist and 100 μM oxotremorine for 2.5 min at 37°C. Controls were incubated similarly except in the absence of any drug treatment, and these sample values are expressed here as 100% ± SEM (*stippled area*). Treatment values are expressed as percentage of untreated control values ± SEM (N = 3 to 5 animals/group).

curve was shifted to the right during veratridine depolarization in a manner similar to that seen during K^+ depolarization.

Discussion

The purpose of these studies was to further characterize the pharmacological properties of the muscarinic receptors mediating the presynaptic modulation of rat cortical ACh release. Previous studies have demonstrated that presynaptic muscarinic receptors can attenuate ACh release from brain slices (Szerb, 1979) as well as peripheral preparations (Briggs and Cooper, 1982); however, few studies have demonstrated consistent modulation in brain synaptosome preparations, and these have usually focused on a superfusion procedure for minimizing the effects of endogenous modulators on ACh release (Raiteri et al. 1984). The batch procedure described in this report (E. St. Onge and E. Meyer, manuscript submitted for publication) also provides consistent results when dilute tissue concentrations are used, and it offers the advantage that many samples can be simultaneously and rapidly assayed for muscarinic release modulation.

Recently, muscarinic receptor heterogeneity has been demonstrated with a variety of muscarinic agonists and antagonists in binding studies (Birdsall et al., 1984). Generally, these receptors have been divided into two types: those blocked selectively by pirenzepine and stimulated by pilocarpine (termed M_1) and those activated selectively by carbachol and blocked allosterically by gallamine (termed M_2). M_2- and M_1-receptors are also found differentially distributed throughout the brain and periphery. Interestingly, recent evidence suggests that M_1-receptors predominate in the rat cerebral cortices, but that M_2-receptors are preferentially located on cholinergic neurons in this tissue and derive from the nucleus basalis, making them likely candidates for mediating presynaptic cholinergic release modulation (Potter et al., 1984).

Our results indicate that the presynaptic muscarinic modulation of [^{3}H]ACh release can be demonstrated in isolated nerve terminals prepared from this region, and that these receptors are the M_2 type. Oxotremorine, ACh, bethanechol, and methacholine modulate [^{3}H]ACh release with different potencies but similar efficacies, suggesting that they can act on the same number of receptors. These four agonists are not selective for either M_1- or M_2-receptors; however, carbachol also modulates [^{3}H]ACh release at low concentrations, whereas pilocarpine does not, which is only consistent with an M_2-mediated modulation. In order to determine whether release modulation was blocked by antagonists that act at M_1- or M_2-receptors, we chose an agonist, oxotremorine, that acts on both receptor types at a concentration high enough to activate low as well as high affinity sites (since M_1 sites may be functionally active only in the low affinity state). However, even under these conditions, and using a tissue that is predominately M_1-laden, we are unable to block modulation with M_1-selective antagonists, such as pirenzepine, unable to block modulation with M_1-selective antagonists, such as pirenzepine or dicyclomine, whereas less specific antagonists completely block modulation. Gallamine, which has a complicated effect on M_2-receptors with respect to inhibiting their agonist binding, blocks modulation only at very high concentrations. Raiteri et al. (1984) similarly found a synaptosomal agonist potency series for ACh release attenuation in which ACh > oxotremorine > carbachol (ED_{50} values from about 1 to 20 μM). This study also showed that pirenzepine was less potent than atropine with respect to blocking

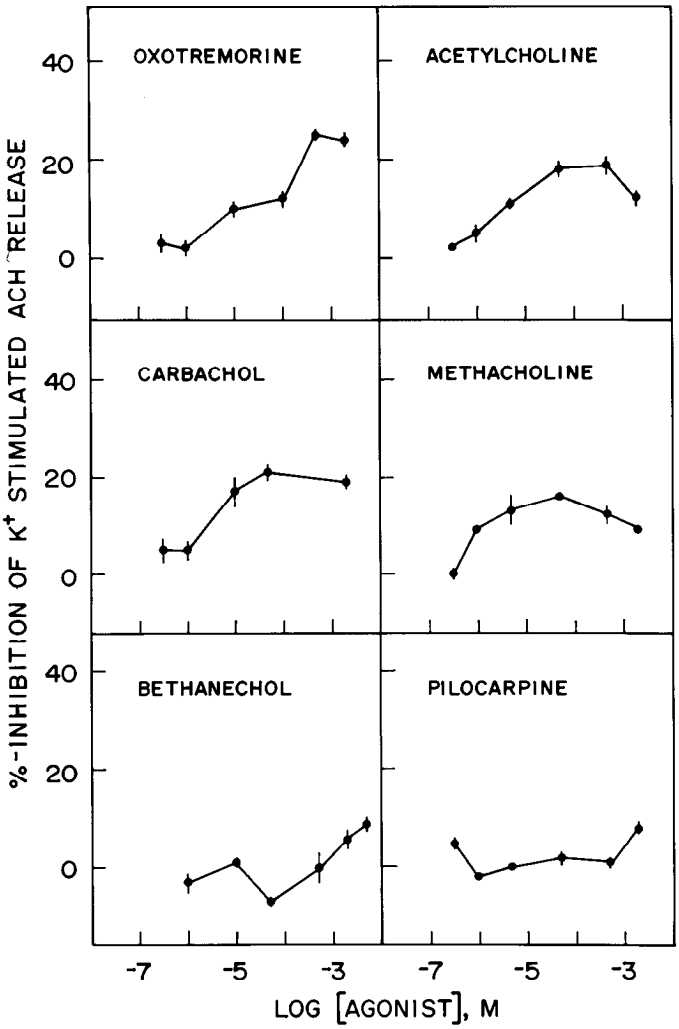

Figure 3. Dose-response curves for muscarinic agonist-induced inhibition of [^{3}H]ACh release in the presence of a depolarizing, 30 mM K^+ concentration. Rat cortical synaptosomes were treated as described in Figure 1, except that KCl was added after the first 30 sec to a final concentration of 30 mM. Control values were determined from samples treated with 30 mM KCl but with no agonist (N = 4 to 5 animals/group). All values are means ± SEM.

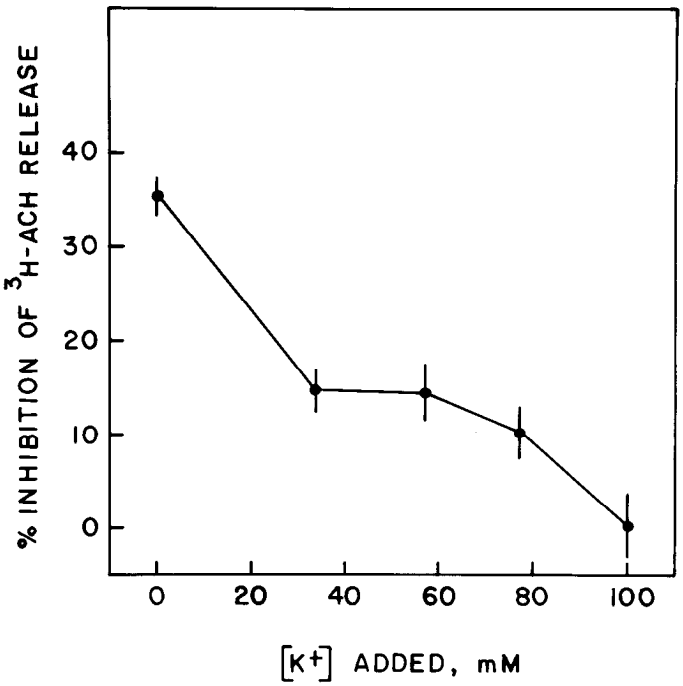

Figure 4. Effects of increasing K^+ concentration on the oxotremorine-induced inhibition of [^{3}H]ACh release. Rat cortical synaptosomes that were preloaded with [^{3}H]ACh were incubated for 30 sec at 37°C in the presence or absence (control value = 100%) of 100 μM oxotremorine; at that time, KCl was added to obtain the specified concentration (in addition to the normal 5.5 mM concentration), and the incubation continued for 2 min more. The [^{3}H]ACh released into the medium was then measured, and the difference in release for each K^+ concentration between oxotremorine-treated and untreated values was determined and expressed here as the mean percentage inhibition of release ± SEM of three animals.

muscarinic modulation; however, pirenzepine was ineffective in our hands at high concentrations (e.g., 100 μM) that inhibited muscarinic release modulation in that study.

M_2-receptor agonist binding can be divided into at least two affinity states: low and high (Birdsall et al., 1984). The ED_{50}s for release modulation by ACh, oxotremorine, and carbachol were all in the low micromolar, high affinity range, which suggests either that the high affinity M_2 agonist binding conformation was selectively responsible for mediating the muscarinic release modulation, that this high affinity site predominated in those synaptosomes in which modulation occurred, or that only a small fraction of low affinity sites need to be occupied to obtain maximum modulation. At present, it is impossible to differentiate between these possibilities because of the heterogeneity of nerve terminals in the brain synaptosome preparation.

M_2 receptor agonist-binding affinity has been shown to be sensitive to osmotic strength, with increasing osmotic strength reducing agonist potency (Potter et al., 1984). However, when synaptosomes were exposed to higher NaCl concentrations, the potency of oxotremorine was unchanged with respect to release modulation, whereas the efficacy of this agonist increased. In contrast, depolarization of the synaptosomes with an equal concentration of KCl instead of NaCl did reduce agonist potency for all of the drugs tested. That this effect of K^+ ions may be due to depolarization and not to K^+

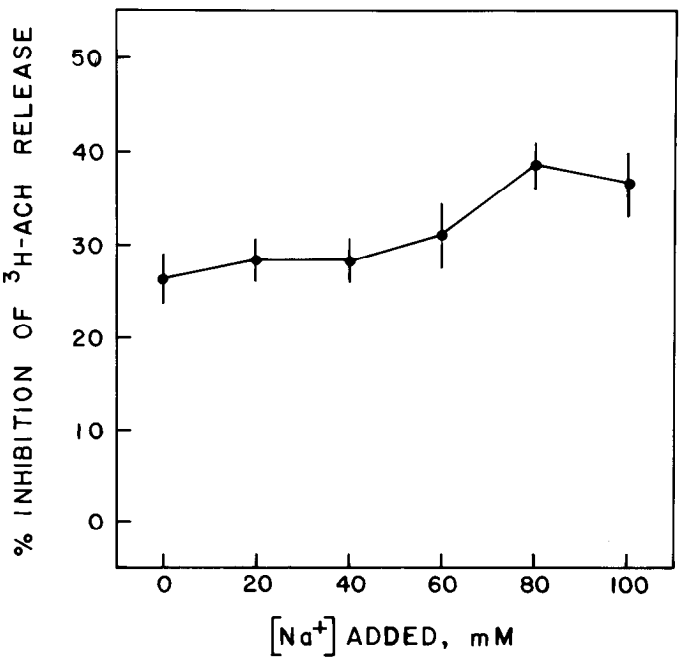

Figure 5. Effects of increasing Na^+ concentrations on the oxotremorine-induced inhibition of [^{3}H]ACh release. Rat cortical synaptosomes were treated as in Figure 4, except that NaCl was added to the specified concentration instead of KCl. All values are means ± SEM of four animals.

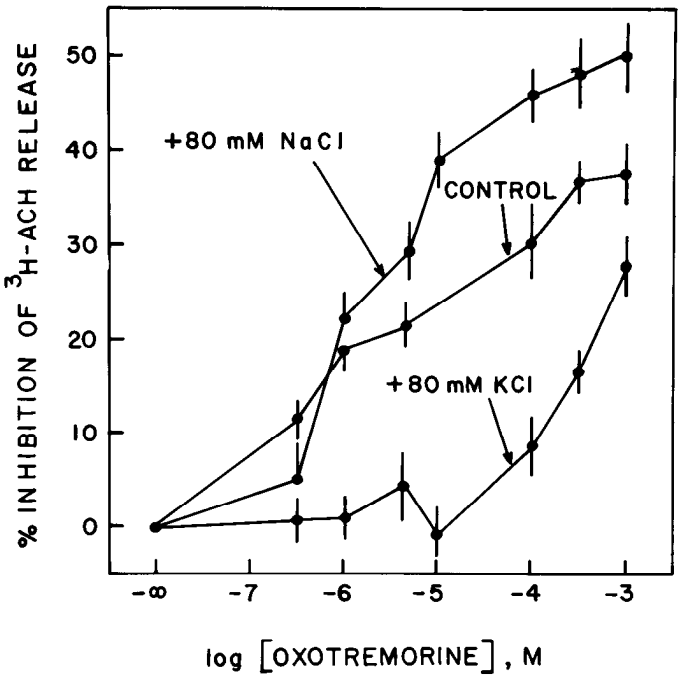

Figure 6. Effects of increasing K^+ or Na^+ concentrations on the dose-response curve for oxotremorine-induced inhibition of [^{3}H]ACh release. Rat cortical synaptosomes were incubated with the specified concentration of oxotremorine for 30 sec; at that time, NaCl or KCl was added for another 2 min, when the release incubation was terminated. The released [^{3}H]ACh was then measured and expressed as in Figure 1, with control values for each group derived from oxotremorine-free samples (N = 4 animals/group).

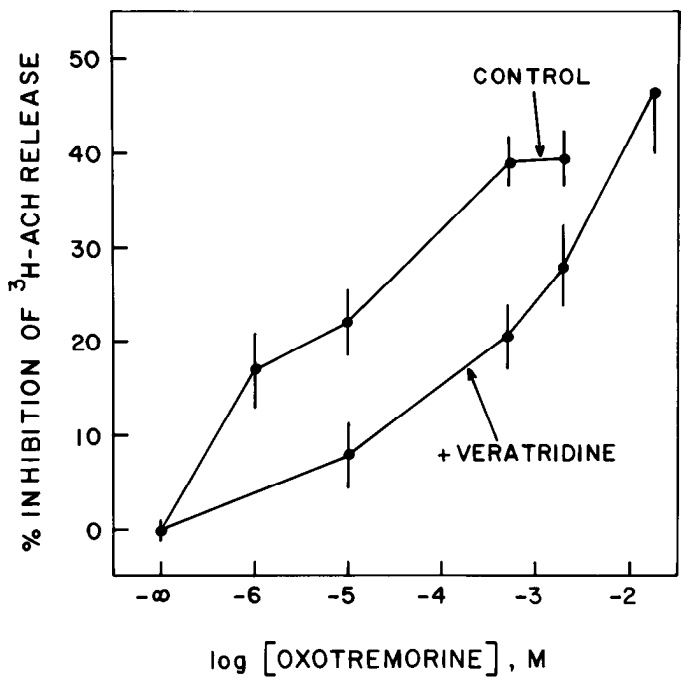

Figure 7. Effects of veratridine on the dose-response curve for oxotremorine-induced inhibition of [^{3}H]ACh release. Synaptosomes were incubated for 2.5 min at 37°C in the presence or absence of 20 μM veratridine plus the specified concentration of oxotremorine. The percentage inhibition of [^{3}H] ACh release was then determined as described in Figure 1, except for veratridine-treated samples, for which control values were oxotremorine-free samples (100%) containing veratridine.

ions per se is suggested by the observations that K^+ ions have no effect on agonist potency in muscarinic binding studies (Birdsall and Hulme, 1983), and our observation that veratridine-induced depolarization induced a similar potency reduction. Further studies are necessary to determine whether K^+ and veratridine depolarization alter receptor-agonist interactions directly or whether secondary processes necessary for ACh release such as increased calcium influx are involved. It is conceivable that depolarization may be one step in the regulation of ACh release modulation, such that muscarinic release inhibition is reduced during intervals of increased neuronal activity and subsequent depolarization; i.e., when maximal transmitter release is required.

High concentrations of ACh or methacholine appeared to reduce the release attenuation observed at intermediate drug concentrations. This effect was not observed by Raiteri et al. (1984) in this preparation, and it was not consistently observed in ours. However, it was blocked by hexamethonium when it was observed, suggesting a nicotinically mediated increase in ACh release under some conditions. Nicotinic stimulation of ACh release has been observed in other tissues, notably in guinea pig myenteric plexus synaptosomes (Briggs and Cooper, 1982); however, we were unable to directly demonstrate modulation with the nicotinic agonist dimethylphenylpiperazinium that elevates release in these plexus synaptosomes.

Taken together, these results are significant for several reasons. First, since only cholinergic terminals can synthesize and release ACh under these conditions, they suggest that presynaptic receptor-mediated ACh release modulation may provide a system for characterizing functional presynaptic M_2-acting drugs. This is in contrast to receptor binding assays that cannot be related easily to function or ascribed pre- or postsynaptically. Second, they indicate that cholinergic transmission may be selectively modulated pharmacologically at the nerve terminal level by drugs that are specific for these M_2-receptors. Along this line, future experiments will be necessary to determine the postsynaptic localization of M_2-receptors in various brain regions, and whether these M_2-receptors can be differentiated from presynaptic M_2-receptors. Third, our results may be important mechanistically, since M_1- and M_2-receptors appear to be differentially transduced into cellular changes; e.g., M_2-receptors in rat brain synaptosomes appear to be coupled to phosphatidylinositol turnover, whereas M_2-receptors are not (Gonzales and Crews, 1984). Along this line, we find no effect of phorbol esters, which increase phosphatidylinositol turnover, on muscarinic ACh release modulation (not shown), suggesting that this turnover is not coupled to M_2-receptor activation and transduction.

Another mechanistic observation involves the apparent influence of membrane potential on agonist potency, since this may provide the nerve terminal with a mechanism for reducing modulation during intervals of intense activity when maximum release and transmission are required, and when maximal transmitter levels are found in the synaptic cleft. Whether this potential-dependent shift in agonist potency is related to GTP-dependent potency shifts observed in receptor binding studies is unclear, as well as whether similar shifts in antagonist potency are also observed during membrane depolarization. We are currently attempting to answer these questions, which are important ones for understanding the regulation of transmission and developing new techniques for modulating it.

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