Subtype-Selective Positive Cooperative Interactions Between Brucine Analogs and Acetylcholine at Muscarinic Receptors: Functional Studies

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ABSTRACT

In radioligand binding studies, it has been reported that brucine, N-chloromethyl brucine, and brucine N-oxide increased the affinity of acetylcholine for M1, M3, and M4 muscarinic receptors, respectively, in a manner consistent with the predictions of the ternary complex allosteric model. We now demonstrate an equivalent ability of these three allosteric agents to modulate the actions of acetylcholine in functional studies in membranes and in whole cells. The enhancing actions of brucine and brucine N-oxide on acetylcholine (ACh) potency at M1 and M4 receptors respectively have been confirmed in A1 (Bruns and Fergus, 1990, Kollias-Baker et al., 1994), A2A-adrenergic (Leppik et al., 1998), and dopamine D2 receptors (Hoare and Strange, 1996) has also been characterized. The first compound that was shown to interact allosterically at muscarinic receptors was gallamine (Clark and Mitchelson, 1976; Stockton et al., 1983), and it satisfies the equilibrium and kinetic predictions of the ternary complex allosteric model (Fig. 1) in both binding (Stockton et al., 1983) and functional (Ehlert, 1988; Lazareno and Birdsall., 1995) studies on M2 receptors. The allosteric site is present on all five muscarinic receptor subtypes (Ellis et al., 1991), and a number of other ligands have been discovered that interact allosterically at muscarinic receptors (for a review, see Lee and El-Fakahany, 1991, Ellis, 1997, Holzgrabe and Mohr, 1998). According to the ternary complex allosteric model, the ac-
tions of an allosteric agent are defined by two parameters, the affinity of the allosteric ligand, X, for the unoccupied receptor, $K_X$, and its cooperativity with the ligand, A, interacting at the other (primary) binding site, $\alpha$ (Fig. 1). In this figure, positive cooperativity is present if $\alpha > 1$. The value of $\alpha$ is not just a characteristic of the allosteric ligand but is dependent on the nature of the other interacting ligand, as has been shown for gallamine (Stockton et al., 1983) and other allosteric ligands (Lazareno et al., 1998; Lazareno and Birdsal, 1995; Jakubik et al., 1997).

In the case of gallamine, all reported $\alpha$ values are <1. Subsequently, alcuronium (Tucek et al., 1990) and strychnine (Lazareno and Birdsal, 1995; Proksa and Tucek, 1995) was shown to exhibit positive cooperativity with the antagonist radioligand $[^3H]$N-methylscopolamine at one or more subtypes of muscarinic receptor ($\alpha > 1$), although these compounds were still negatively cooperative with ACh. It should be noted that there is a special importance of the value of the cooperativity with ACh in that any therapeutic effect of a drug as an allosteric agent at muscarinic receptors (or any other receptor) is defined by its cooperativity with the endogenous neurotransmitter.

We are interested in compounds that are positively cooperative with ACh at muscarinic receptors and that may be of use in, for example, the treatment of the cognitive deficits in the earlier stages of Alzheimer’s disease. We have discovered that brucine and some analogs (Fig. 2) are allosteric agents at muscarinic receptors and exhibit positive cooperativity at one or more muscarinic receptor subtypes (Birdsall et al., 1997), a result that has been confirmed for brucine itself (Jakubik et al., 1997). These brucine derivatives satisfy the equilibrium and kinetic predictions of the ternary allosteric model in binding studies (Lazareno et al., 1998). In this report, we examine whether the qualitative and quantitative predictions of subtype selectivity and cooperativity, derived from the binding studies of brucine and its analogs, can be observed in a variety of functional studies on muscarinic receptors.

**Experimental Procedures**

**Materials.** Guanosine-5’-O-[(3-32)S]triphosphate ($[^3S]$GTP$\gamma$S) was from DuPont/NEN (Hounslow, Middlesex, UK). $[^32]$P[GTP was from Amersham International (Cardiff, Wales). Saponin, GDP, brucine sulfate, ACh chloride, Fura 2-acetoxymethyl ester, 5,5’-dithiodiobi(2-nitrobenzoic acid), 9-amino-1,2,3,4-tetrahydroacridine (taclidine), and acetylthiocholine were from Sigma Chemical (Poole, Dorset, UK). Brucine-$N$-oxide hydrate was from Aldrich Chemical Co. (Gillingham, Dorset, UK). Pertussis toxin was from Calbiochem (Nottingham, UK). N-Chloromethylbrucine chloride was synthesized from the reaction of brucine with dichloromethane.

**Cell Culture and Membrane Preparation.** Chinese hamster ovary (CHO) cells stably expressing cDNA encoding human muscarinic $M_2–M_5$ receptors were generously provided by Dr. N. J. Buckley (University College, London). (The nomenclature used in this report is that approved by the IUPHAR Committee on Receptor Nomenclature and Drug Classification (Caulfield and Birdsal, 1998).) The cells were grown in a minimal essential medium (GIBCO, Grand Island, NY) containing 10% (v/v) newborn calf serum, 50 units/ml penicillin, 50 $\mu$g/ml streptomycin, and 2 mM glutamine at 37°C under 5% CO$_2$. Cells were grown to confluence and harvested by scraping in a hypotonic medium (20 mM HEPES plus 10 mM EDTA, pH 7.4). Membranes were prepared at 0°C by homogenization with a Polytron followed by centrifugation (40,000g; 15 min), washed once in 20 mM HEPES plus 0.1 mM EDTA, pH 7.4, and stored at −70°C in the same buffer at protein concentrations of 2 to 5 mg/ml. Protein concentrations were measured with the Bio-Rad reagent using BSA as the standard. The yields of receptor varied from batch to batch but were approximately 5, 1, 7, 2, and 1 pmol/mg total membrane protein for the $M_1–M_5$ subtypes, respectively.

**GTP$\gamma$S Assay.** Membranes were suspended in a buffer containing 20 mM HEPES, 100 mM NaCl, and 10 mM MgCl$_2$, pH 7.4, at a protein concentration of 25 to 50 $\mu$g/ml. To the membrane suspension on ice was added the appropriate concentration of GDP followed by $[^3S]$GTP$\gamma$S (final concentration 100 $\mu$M). Then, 1 ml aliquots were added to polystyrene tubes (5 ml) containing ACh and additional allosteric ligands as appropriate. Incubations were at 30°C for 30 min. The samples were filtered over glass fiber filters (Whatman GF/B) using a Brandel cell harvester and washed with 2×3 ml of water. The filter disks were extracted overnight with 3 ml of scintillant (and counted by liquid scintillation spectrometry at an efficiency of about 97%). Assays were conducted in duplicate, with each set of replicates filtered together. The concentrations of GDP used in these assays were 10$^{-7}$ M for $M_1$ and $M_2$ receptors and 10$^{-6}$ M for $M_3$ and $M_4$ receptors. In some assays, saponin (10 $\mu$g/ml) was present. This increases the signal and the signal-to-noise ratio in such assays without substantially affecting the ACh potency (Cohen et al., 1995; Lazareno, 1997).

**GTPase Assay.** Membranes were suspended in a buffer (0.1 ml) containing 20 mM HEPES, 100 mM NaCl, 5 mM MgCl$_2$, and 1 mM ATP, pH 7.4, at a protein concentration of 50 $\mu$g/ml. To the membrane suspension was added [y$^{32}$P]GTP (final concentration 10–100 nM). Incubations were at 30°C for 30 min, after which the reaction was stopped by the addition of 0.75 ml of a slurry of 5% charcoal in 0.5% Triton X-100 (2 min). Labeled phosphate was counted for radioactivity. Assays were conducted in duplicate.

**cAMP Assay.** CHO cells expressing M$_2$ receptors were detached from the culture flasks by brief exposure to trypsin/EDTA solution and washed twice with a solution containing 118 mM NaCl, 1.8 mM CaCl$_2$, 2.7 mM KCl, 0.81 mM MgSO$_4$, 5.6 mM Na$_2$HPO$_4$, 50 mM glucose, 10 mM HEPES, pH 7.4, and penicillin, 50 $\mu$g/ml streptomycin, and 2 mM glutamine at 37°C under 5% CO$_2$. Cells were grown to confluence and harvested by scraping in a hypotonic medium (20 mM HEPES plus 10 mM EDTA, pH 7.4). Membranes were prepared at 0°C by homogenization with a Polytron followed by centrifugation (40,000g; 15 min), washed once in 20 mM HEPES plus 0.1 mM EDTA, pH 7.4, and stored at −70°C in the same buffer at protein concentrations of 2 to 5 mg/ml. Protein concentrations were measured with the Bio-Rad reagent using BSA as the standard. The yields of receptor varied from batch to batch but were approximately 5, 1, 7, 2, and 1 pmol/mg total membrane protein for the $M_1–M_5$ subtypes, respectively.

**Fig. 2.** Formulas of brucine (R = H, shown here as the protonated species), N-chloromethyl brucine (R = CH$_2$Cl), and brucine $N$-oxide (R = O$^-$).
glucose, 0.5 mM 3-isobutyl-1-methylxanthine, and 10 mM HEPES (pH 7.4). The cells were suspended in the same medium at a density of $5 \times 10^7$ cells/ml. Aliquots of the cell suspension (0.1 ml) were incubated with various concentrations of ACh with or without brucine (100 $\mu$M) at 37°C for 10 min. The reaction was stopped by adding 1 N HCl (final concentration 0.1 N). cAMP levels in each sample were measured with a cAMP enzyme/immunoassay system (Amersham International) after acetylation of the samples with acetic anhydride. Assays were conducted in triplicate.

**Ca$^{2+}$ Assay.** CHO cells expressing $M_1$ receptors were detached from the culture flask by brief exposure with trypsin/EDTA solution and loaded with Fura-2 acetoxymethyl ester (5 $\mu$M) at 37°C for 30 min in 10 ml of α-minimal essential medium containing 10% newborn calf serum. Fura-2-loaded cells were washed twice with 10 ml of Ca$^{2+}$-free Locke’s solution by centrifugation. The cells were suspended in a small volume of Ca$^{2+}$-free Locke’s solution and kept on ice. An aliquot of the cells was incubated in 2 ml of Locke’s solution containing 2.3 mM Ca$^{2+}$ at 37°C, and the fluorescence at 510 nm, which results from the excitation at 340 and 380 nm, was recorded with a fluorescence spectrophotometer (F-2000; Hitachi). Ca$^{2+}$ concentrations were calculated automatically. The magnitude of the Ca$^{2+}$ signal produced by a given concentration ACh slowly decreased (typically by 50–70%) over the 3-h time course of an experiment. Therefore, the response to 3 $\mu$M ACh, a concentration producing a maximal signal, was measured every four or five trials, and the magnitude of the responses produced by different concentrations of ACh was normalized to the interpolated maximal response at the time of measurement to give the dose-response curves of the type shown in Fig. 4B. In general, the effects of any given submaximal concentration of ACh were measured at least two times within an experiment.

**Smooth Muscle Preparation.** The experiments were performed on 5-cm strips of male guinea pig ileum suspended in Tyrode’s solution and bubbled with 95% O$_2$ and 5% CO$_2$. The bath (60 ml) was kept at 37°C. The preparation was coaxially stimulated by rectan-

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**Fig. 3.** Enhancement by brucine of ACh potency at $M_1$ receptors in assays of function in membranes. A, brucine (100 $\mu$M) increased the potency of ACh to stimulate [35S]GTPgS to G proteins in $M_1$-CHO cell membranes. In this experiment, the EC$_{50}$ value for ACh decreased from 2.8 $\mu$M (○) to 0.9 $\mu$M (■) without significantly affecting the basal response or maximal stimulation. B, enhancement of ACh-stimulated [35S]GTPgS binding by brucine in pertussis toxin-treated $M_1$-CHO cell membranes in the presence of saponin. In the control membranes, the potency of ACh in the presence (∇) of brucine (10 $^{-4}$ M) was enhanced 2.1-fold relative to the absence of brucine (○) without changing the Hill slope of the dose-response curve (0.7). The effect of pertussis toxin treatment was to increase ACh potency 8-fold in both the presence (∇) and absence (○) of brucine (10 $^{-4}$ M) such that brucine retained the same enhancing action. The Hill slope of the dose-response curves for the pertussis toxin-treated membranes increased to 1.0. This result was reproduced in two other experiments conducted in the presence and absence of saponin (mean enhancement, 1.8 ± 0.2; $n = 3$).

**Fig. 4.** Dose-response curves for the potentiation by brucine of ACh whole-cell $M_1$ muscarinic receptor responses. A, brucine (10 $^{-4}$ M) enhanced the potency of ACh to increase cAMP accumulation in $M_1$-CHO cells by 2.6-fold. B, dose-response curves generated from the data shown in Fig. 5 and additional data from the same experiment, normalized to the response to 10 $^{-6}$ M ACh alone, show that brucine (100 $\mu$M) produced a 3.0-fold increase in ACh potency.
gular current pulses (0.1 Hz, 1-ms duration, 1.0–1.5 V), conditions chosen to generate a submaximal contraction. After an established contraction was obtained, compounds were added cumulatively at intervals of 3 min.

**Acetylcholinesterase Assay.** The isolated guinea pig ileum was homogenized in a pH 8 phosphate buffer (2:1 w/v), diluted 200-fold in the same buffer, and filtered through a 0.45-μm filter. The filtrate was incubated with N-chloromethyl brucine (2–600 μM) or tacrine (3 μM) and acetylthiocholine (90 μM) for 30 min at 37°C. Thiocholine was assayed spectrophotometrically at (412 nm) using 5,5'-dithio-bis(2-nitrobenzoic acid). (Ellman et al., 1961)

**Data Analysis.** Data were fitted using equations derived previously from the ternary allosteric complex model (Lazareno et al., 1995, 1998) and nonlinear regression analysis using the fitting procedure in SigmaPlot (SPSS, Ekrath, Germany). This procedure allows the use of two or more independent variables (e.g., the concentration of two drugs). Unless otherwise stated, the results are presented as mean ± S.E.M. (n represents the number of independent experiments). The enhancement by brucine and its analogs of ACh potency in the (paired) functional assays was assessed by a single-tailed paired-sample t test. All the enhancements in potency reported here were significant at the 1% level except for one set of experiments, where P < .05.

**Results**

**Allosteric Enhancement of M<sub>1</sub> Receptor Function in Membranes and Whole Cells.** We reported previously that in binding studies, brucine exhibits a 1.6 ± 0.1-fold positive cooperativity with ACh at M<sub>1</sub> receptors (Lazareno et al., 1998). The positive cooperativity at M<sub>1</sub> receptors has been confirmed in [35S]GTP<sub>γ</sub>S functional assays in membranes where the ability of an agonist (in these experiments, ACh) to increase the rate of binding of [35S]GTP<sub>γ</sub>S to G proteins was measured under the same ionic conditions as the binding studies (Lazareno et al., 1993, Lazareno and Birdsall, 1993). In the experiment shown in Fig. 3A, ACh stimulated the binding of [35S]GTP<sub>γ</sub>S with an EC<sub>50</sub> value of 2.8 μM. In the presence of brucine (10<sup>-2</sup> M), the ACh potency increased 3-fold to 0.9 μM. As illustrated here, neither the basal level of [35S]GTP<sub>γ</sub>S binding nor the maximal level of stimulation was changed significantly by the presence of brucine. The threshold concentration of brucine for observing an enhancement of ACh potency was about 30 μM (data not shown). We have observed in experiments with strychnine (Lazareno et al.,

**Fig. 5.** Allosteric enhancement by brucine of a whole-cell M<sub>1</sub> Ca<sup>2+</sup> response to ACh. ACh (10<sup>-8</sup> to 10<sup>-6</sup> M) produced a dose-dependent increase in intracellular Ca<sup>2+</sup> concentration levels in M<sub>1</sub>-CHO cells (A–C). The responses to 10<sup>-8</sup> and 10<sup>-7</sup> M ACh are significantly potentiated by 10<sup>-4</sup> M brucine (D and E), whereas a much smaller effect on the response to 10<sup>-6</sup> M ACh is observed (F). The potentiation, however, was not dependent on the order of addition of brucine and ACh (G), and the intracellular Ca<sup>2+</sup> concentration elevation in the presence and absence of brucine was completely blocked by the muscarinic antagonist 3-quinuclidinyl benzilate (1 μM) (H). These results are from a single representative experiment that was repeated three times.
an artifact mediated by an effect on Gi/o proteins or a change of ACh potency by brucine is not an allosteric effect but that there was a slight chance that the observed enhancement was not observed with the brucine analogs, it was considered 1993, Lazareno, 1997). Although a reduction in basal activity was known from preliminary experiments to block essentially all G\textsubscript{\alpha} protein function. These \textsuperscript{[35S]}GTP\textsubscript{\gamma}S assays were carried out in the presence of saponin to enhance the signal-to-noise ratio. In the control membranes, the ACh dose-response curve had a Hill slope of about 0.7 and its potency was increased 2.1-fold by 10\textsuperscript{-4} M brucine without significantly affecting the slope. The effect of pertussis toxin treatment was to reduce basal binding \textasciitilde50\%, to increase ACh potency 8-fold, and to increase the Hill slope of the curves to 1. The enhancement of ACh potency by brucine (1.9 \pm 0.1-fold, n = 2) was retained in the pertussis toxin-treated membranes, indicating that brucine equally enhances the ability of ACh-M\textsubscript{1} receptor complexes to activate both G proteins sensitive to and those insensitive to pertussis toxin treatment.

Brucine also enhanced the ability of ACh to generate M\textsubscript{1} receptor-linked whole-cell responses. At a concentration of 10\textsuperscript{-4} M, it potentiated the EC\textsubscript{50} value of ACh-stimulated cAMP accumulation in M\textsubscript{1}-CHO cells by 2.4 \pm 0.4-fold (n = 4) (Fig. 4A) without affecting basal levels or the maximal response. It also potentiated the ability of ACh to elevate intracellular Ca\textsuperscript{2+} concentration in the same M\textsubscript{1}-CHO cell line (Fig. 4B). Again, there was a lack of effect of brucine on the maximum response. The observed enhancement of ACh potency (2.5 \pm 0.2-fold, n = 3) was comparable to the cooperativity observed in the membrane assays of ACh binding and function and in the cAMP whole-cell assay of function.

The enhancement by brucine of the amplitude of the Ca\textsuperscript{2+} signaling response to submaximal concentrations of ACh (10\textsuperscript{-8} and 10\textsuperscript{-7} M) is clearly illustrated in Fig. 5 (compare A and B with D and E). No significant effect of brucine was observed in the absence of ACh (Fig. 5, D–F), and the size of the response was not dependent on the order of addition of ACh and brucine (compare D with G). All responses were reversible and blocked by the muscarinic antagonist 3-quinuclidinylbenzilate (10\textsuperscript{-6} M) (Fig. 5H).

**Allosteric Enhancement of M\textsubscript{3} Receptor Function in Membranes by N-Chloromethyl Brucine and Its Actions on Other Subtypes.** N-Chloromethyl brucine exhibits a different selectivity from that of brucine and brucine N-oxide. It enhances the binding of ACh 3.3-fold at M\textsubscript{3} receptors but not at the other subtypes, being very slightly negatively cooperative at M\textsubscript{1}, strongly negative at M\textsubscript{2} receptors, and essentially neutrally cooperative at M\textsubscript{4} receptors (Lazareno et al., 1998).

Because the observed magnitude of positive cooperativity and the range of values of cooperativity between the subtypes in binding studies is larger than observed for the other two compounds, detailed dose-response curves were generated for the effects of N-chloromethyl brucine on ACh-stimulated \textsuperscript{[35S]}GTP\textsubscript{\gamma}S binding to membranes of M\textsubscript{1}–M\textsubscript{4} transfected cells (Fig. 7). N-Chloromethyl brucine did not affect the basal or maximal responses or the slope of the ACh dose-response curves. The shifts of the curves are shown in the insets. No significant dose-dependent shifts in the dose-response curves at M\textsubscript{1} and M\textsubscript{4} receptors were observed (range of pEC\textsubscript{50} values 10\textsuperscript{-2} M brucine N-oxide in a GTPase assay of function (Fig. 6). In measures of ACh-stimulated GTP\textsuperscript{\gamma}S binding at M\textsubscript{4} receptors, the EC\textsubscript{50} value for ACh is about 2 to 3 \times 10\textsuperscript{-7} M (Lazareno et al., 1993; Lazareno and Birdsall, 1993); significant enhancements of the actions of a submaximal single concentration ACh (10\textsuperscript{-7} M) were observed using 3 \times 10\textsuperscript{-4} or 10\textsuperscript{-3} M brucine N-oxide. These were equivalent to 1.5- to 2.9-fold (range, n = 10) increases in potency. In analogous experiments to those illustrated in Fig. 3A, 3 \times 10\textsuperscript{-4} M brucine N-oxide increased ACh potency at M\textsubscript{4} receptors by 1.9 \pm 0.3-fold (n = 2, data not shown).

In binding studies, brucine N-oxide is positively cooperative with ACh at M\textsubscript{3} receptors, neutral at M\textsubscript{1} receptors, and weakly negatively cooperative at M\textsubscript{2} receptors (Lazareno et al., 1998). This qualitative profile was also observed in \textsuperscript{[35S]}GTP\textsubscript{\gamma}S functional studies on these subtypes (data not shown).

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ues, 5.80–5.97 and 6.80–6.97 at M1 and M4 receptors, respectively). The results at M2 and M3 receptors illustrate clearly the qualitatively different effects of chloromethylbrucine at these subtypes. Increasing concentrations of N-chloromethylbrucine progressively shift the ACh dose-response curve for M2 receptors to the right of the control (dashed) curve, whereas the M3 curve moves to the left as ACh becomes more potent. Furthermore, these data are capable of being analyzed by the allosteric model, with the curves in Fig. 7 being the fits derived from nonlinear regression analysis. The calculated cooperativities with ACh in this experiment were 0.02 and 4.6 at M2 and M3 receptors, respectively. There is a good agreement between the affinities of N-chloromethylbrucine and its cooperativities with ACh at M2 and M3 receptors, as determined by analyses of the binding and functional data by the allosteric model (Table 1).

The enhancing actions of N-chloromethyl brucine were also examined in a M3 whole tissue preparation, the isolated guinea pig ileum. N-Chloromethyl brucine (20–200 \( \mu \text{M} \)) produced a 1.8- to 2.6-fold increase in the submaximal contractions produced by a low concentration (2 \( \times 10^{-9} \) M) of ACh \((n = 4, \text{data not shown})\) but had no effect on the maximal contractions generated using higher concentrations of ACh (2 \( \times 10^{-7} \) to 2 \( \times 10^{-6} \) M). It was also possible to observe a dose-dependent potentiation by N-chloromethyl brucine (2–200 \( \mu \text{M} \)) of the electrically stimulated contraction of isolated strips of guinea pig ileum, a whole tissue response mediated via ACh release and M3 receptor activation (Fig. 8). The threshold concentration of N-chloromethyl brucine for the potentiation of the submaximal stimuli was 2 to 10 \( \mu \text{M} \) and a more than 3-fold enhancement was observed at 200 \( \mu \text{M} \). The potentiation was similar to that shown by eserine (4–400 nM, data not shown) but was not caused by acetylcholinesterase inhibition because N-chloromethyl brucine (2–600 \( \mu \text{M} \)) did not inhibit the acetylcholinesterase in homogenates of guinea pig ileum: the enzyme activity was 104 ± 4% \((n = 15)\) of the control value, whereas in the presence of tacrine \((10^{-6} \text{ M})\), a positive control, the residual activity was 3 ± 1% \((n = 3)\). The electrically stimulated responses in this tissue were abolished by atropine (300 nM) but were unaffected by the nicotinic antagonist hexamethonium (40 \( \mu \text{M} \)). N-Chloromethyl brucine (2–200 \( \mu \text{M} \)) failed to affect the electrically stimulated contractions in the rat phrenic nerve preparation that are mediated via nicotinic receptors: these contractions were potentiated by eserine (400 nM). The potentiation by N-chloromethyl brucine of the field-stimulated contractions might reflect a presynaptic inhibition of ACh affinity at an M2 autoreceptor rather than a postsynaptic enhancement of M3.

**Fig. 7.** Allosteric modulation by N-chloromethyl brucine of ACh-stimulated \([^{35}\text{S}]\text{GTP} \gamma \text{S} \) binding at M1–M4 receptors.

At M1 and M4 receptors, there was no dose-dependent change in the pEC\(_{50}\) value of ACh within the experiment illustrated here, and in additional experiments, the mean pEC\(_{50}\) values at the two subtypes was 5.87 ± 0.09 and 6.90 ± 0.09 \((\text{mean ± range/2, } n = 5)\). At M2 and M3 receptors, the lines represent the simultaneous fits of the data to the ternary allosteric model. The basal and maximal responses were constrained to be the same for a given subtype. For M2 and M3 receptors, respectively, the calculated values of the pEC\(_{50}\) for ACh in the absence of allosteric ligand were 7.20 and 5.29; the slopes of the ACh dose-response curves were 0.81 and 0.87; the log affinities of N-chloromethylbrucine were 4.30 and 3.86; and the cooperativity with ACh was 0.02 and 4.6. The averaged values from five experiments are summarized in Table 1. At M4 receptors, the lines represent the simultaneous fits of the data to the ternary allosteric model. The basal and maximal responses were constrained to be the same for a given subtype. For M4 receptors, the calculated values of the pEC\(_{50}\) for ACh in the absence of allosteric ligand were 7.20 and 5.29; the slopes of the ACh dose-response curves were 0.81 and 0.87; the log affinities of N-chloromethylbrucine were 4.30 and 3.86; and the cooperativity with ACh was 0.02 and 4.6. The averaged values from five experiments are summarized in Table 1. At M4 receptors, the lines represent the simultaneous fits of the data to the ternary allosteric model. The basal and maximal responses were constrained to be the same for a given subtype. For M4 receptors, the calculated values of the pEC\(_{50}\) for ACh in the absence of allosteric ligand were 7.20 and 5.29; the slopes of the ACh dose-response curves were 0.81 and 0.87; the log affinities of N-chloromethylbrucine were 4.30 and 3.86; and the cooperativity with ACh was 0.02 and 4.6. The averaged values from five experiments are summarized in Table 1. At M4 receptors, the lines represent the simultaneous fits of the data to the ternary allosteric model. The basal and maximal responses were constrained to be the same for a given subtype. For M4 receptors, the calculated values of the pEC\(_{50}\) for ACh in the absence of allosteric ligand were 7.20 and 5.29; the slopes of the ACh dose-response curves were 0.81 and 0.87; the log affinities of N-chloromethylbrucine were 4.30 and 3.86; and the cooperativity with ACh was 0.02 and 4.6. The averaged values from five experiments are summarized in Table 1. At M4 receptors, the lines represent the simultaneous fits of the data to the ternary allosteric model. The basal and maximal responses were constrained to be the same for a given subtype. For M4 receptors, the calculated values of the pEC\(_{50}\) for ACh in the absence of allosteric ligand were 7.20 and 5.29; the slopes of the ACh dose-response curves were 0.81 and 0.87; the log affinities of N-chloromethylbrucine were 4.30 and 3.86; and the cooperativity with ACh was 0.02 and 4.6. The averaged values from five experiments are summarized in Table 1.
receptors. However, in contrast to N-chloromethyl brucine, the M<sub>2</sub>-selective antagonist methoctramine (1.4 nM to 4 μM) did not enhance the contractions at any of these concentrations (n = 4, data not shown), but some inhibition was observed at concentrations of methoctramine above 40 nM. The enhancement in Fig. 8 therefore probably is not a presynaptic effect but is due to N-chloromethyl brucine acting as an allosteric enhancer at postsynaptic M<sub>3</sub> receptors.

**Discussion**

We examined the allosteric actions of brucine and two derivatives, brucine N-oxide and N-chloromethyl brucine, in several functional assays on a number of muscarinic receptor subtypes. These compounds were chosen from a range of brucine and strychnine derivatives (Birdsall et al., 1997, Lazareno et al., 1998, Gharagozloo et al., 1999) because of their ability to selectively enhance the binding of ACh to different muscarinic receptor subtypes; furthermore, the allosteric effects of these compounds in binding studies satisfied the equilibrium and kinetic predictions of the allosteric ternary complex model (Fig. 1) (Lazareno et al. 1995, 1998).

We have demonstrated that in a variety of membrane and whole-cell assays of muscarinic receptor function, brucine, N-chloromethyl brucine, and brucine N-oxide are allosteric enhancers at M<sub>1</sub>, M<sub>2</sub>, and M<sub>3</sub> receptors, respectively. In general, the compounds do not affect either the basal activity or function in the presence of maximally effective concentrations of ACh (Figs. 3, A and B, 4, A and B, 5, and 7). Only the actions of submaximal concentrations of ACh are affected. This illustrates the prediction of the simple allosteric model that an allosteric drug will have no pharmacological action unless the endogenous ligand for the receptor is present.

As a consequence, we find no evidence in our functional studies reported here (or elsewhere, e.g., Lazareno et al., 1995, Ehlert, 1988) that the allosteric agents, including galamine and strychnine and the brucine analogs, activate muscarinic receptors. This is in contrast to the results reported by Jakubik et al. (1996), but we have not carried out the same assays of function.

Brucine analogs and most other reported allosteric agents (but not all, such as obidoxime; Ellis and Seidenberg, 1992) slow down the association and dissociation kinetics of [3H]ACh at M<sub>2</sub> and M<sub>3</sub> receptors measured in binding and functional studies (n = 5).

<table>
<thead>
<tr>
<th>Subtype</th>
<th>Binding Studies&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Function</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Log Affinity</td>
<td>Cooperativity with ACh</td>
</tr>
<tr>
<td>M&lt;sub&gt;2&lt;/sub&gt;</td>
<td>4.22 ± 0.11</td>
<td>0.09 ± 0.02</td>
</tr>
<tr>
<td>M&lt;sub&gt;3&lt;/sub&gt;</td>
<td>3.66 ± 0.13</td>
<td>3.3 ± 0.2</td>
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<sup>a</sup> The binding data are from Lazareno et al. (1998).

**Fig. 8.** A, N-Chloromethyl brucine, in a dose-dependent manner, enhanced the field-stimulated contractions of isolated guinea pig ileum strips. The contractions were inhibited by atropine (30 nM). B, histogram of the percentage enhancement of contraction produced in four independent experiments of the type illustrated in A. Data are expressed as mean ± S.E.M.
to fit simultaneously sets of ACh dose-response curves in the presence of several concentrations of allosteric agent to the allosteric ternary complex model and to obtain well defined estimates of $K_A$ and $\alpha$. In the case of N-chloromethyl brucine, however, it was possible to demonstrate clearly that its positive and negative allosteric effects on ACh-stimulated GTP$\gamma$S binding, mediated via $M_3$ and $M_4$ receptors respectively, were well fitted by the simple allosteric model (Fig. 7). Furthermore, the parameters defining the allosteric action in binding and function were in good agreement (Table 1). These findings illustrate that it is possible to have a compound exhibiting the opposite pharmacological actions of an allosteric enhancer ($\alpha = 3–4$) and inhibitor ($\alpha = 0.02–0.09$), a ratio of about 60, for two closely related receptor subtypes.

A further and, paradoxically, possibly more important feature of the functional studies depicted in Fig. 7 is the immeasurably small effects of up to 300 $\mu$M N-chloromethyl brucine on ACh function at $M_1$ and $M_2$ receptors. This is true despite the fact that 10 to 300 $\mu$M N-chloromethyl brucine has dramatic slowing effects on the dissociation rate constant of $[^3H]N$-methylscopolamine from these receptor subtypes (as well as from $M_2$ and $M_3$ receptors) (Lazareno et al., 1998). The lack of effect on ACh function is entirely compatible with the fact that in radioligand binding studies, N-chloromethyl brucine exhibits neutral cooperativity with ACh at $M_4$ receptors ($\alpha = 1.0 \pm 0.1$) and only very slight negative cooperativity with ACh at $M_1$ receptors (Lazareno et al., 1998).

This result illustrates the importance of neutral cooperativity in pharmacological selectivity. Despite the fact that N-chloromethyl brucine is binding to $M_4$ receptors at the same concentrations as it is producing its enhancing actions at $M_4$ receptors and its allosteric inhibitory actions at $M_2$ receptors, it has no action at $M_4$ receptors. We used the term “absolute subtype selectivity” for a positive (or negative) cooperative action at one subtype and the lack of pharmacological action associated with neutral cooperativity at another subtype (Birdsall et al., 1997; Lazareno et al., 1998). Such selectivity is not available to agonists and competitive antagonists, where affinity and/or efficacy is the determinant of relative subtype selectivity. In the case of allosteric agents, both affinity and cooperativity are independent parameters that determine pharmacological action and selectivity.

Finally, we extended our study to the demonstration of allosteric enhancement of muscarinic receptor function in a whole tissue. Again, we chose N-chloromethyl brucine as the allosteric enhancer at $M_3$ receptors because of its large positive cooperativity with ACh. The tissue model was the guinea pig ileum strip in which electrical stimulation causes the release of ACh and the consequent contraction of smooth muscle via stimulation of $M_3$ receptors. It was possible to demonstrate a potentiation by N-chloromethyl brucine of contractions elicited by submaximal (but not maximal) electrical stimulation or exogenous ACh application (Fig. 8). Appropriate controls eliminated any contribution to the contractile response or the actions of N-chloromethyl brucine by nictinic ACh receptors, presynaptic $M_2$ inhibitory autoreceptors, or acetylcholine-terase inhibition.

These results suggest the feasibility that a selective allosteric enhancer, acting at a specific muscarinic subtype, could enhance subnormally functioning cholinergic synapses in the central nervous system while having less or no action at normally functioning synapses. The existence of a regional cholinergic deficit in the earlier stages of Alzheimer’s disease and the association of muscarinic receptors with memory and cognition suggest one possible therapeutic area for muscarinic allosteric enhancers.

**Note Added in Proof.** It has been reported recently that brucine modulates the action of synthetic agonists on [3H]ACh release in rat striatal slices by an action at $M_4$ receptors (Dolezal V and Tucek S (1998) Br J Pharmacol 124:1213–1218).

### References


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