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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 M_1, M_3, and M_4 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 M_1 and M_4 receptors respectively have been confirmed in guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate, GTPase, cAMP, and intracellular Ca^{2+} mobilization assays of function. In general, neither the basal nor the maximally stimulated response to ACh is affected. The subtype-selective allosteric effects of *N*-chloromethyl brucine on M_2 and M_3 receptors were shown to be qualitatively and quantitatively the same in guanosine-5′-*O*-(3-[^{35}S]thio)triphosphate functional assays, in terms of both its affinity and cooperativity with ACh, as those found in binding assays. Neutral cooperativity of *N*-chloromethyl brucine with ACh on M_4 receptor function was also observed, thereby demonstrating its "absolute subtype selectivity": a lack of action at any concentration at M_4 receptors and an action at M_2 and M_3 receptors. The enhancing action of *N*-chloromethyl brucine on neurogenically released ACh binding at M_3 receptors was also detected in whole tissue as an increased contraction of the isolated guinea pig ileum to submaximal electrical stimulation. In conclusion, these functional studies confirm that brucine analogs are allosteric enhancers of ACh affinity at certain muscarinic receptor subtypes.

Exogenous receptor ligands can increase receptor function either directly, by causing the receptor to adopt an active conformation, or indirectly, by increasing the efficacy or affinity of the endogenous ligand for its receptor. This indirect (allosteric) mechanism is the molecular basis for the therapeutic actions of benzodiazepine tranquilizers and other ligands that enhance γ-aminobutyric acid actions at γ-aminobutyric acid-gated receptor channels (Macdonald and Olsen, 1994; Costa and Guidotti, 1996). Although allosteric phenomena are relatively common in the superfamily of ion-channel coupled receptors, there is, to our knowledge, no drug that produces its therapeutic action via an allosteric mechanism at G protein-coupled receptors.

The first and most thoroughly studied allosteric site on a G protein-coupled receptor is that on muscarinic acetylcholine (ACh) receptors (for recent reviews, see Birdsall et al., 1995, Tucek and Proska, 1995, Ellis, 1997, Christopoulos et al., 1998, Holzgrabe and Mohr, 1998); allosterism at adenosine A_1 (Bruns and Fergus, 1990, Kollias-Baker et al., 1994), α_{2A}-adrenergic (Leppik et al., 1998), and dopamine D_2 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 M_2 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-

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A brief description of some of the results has been reported previously (Birdsall et al., 1997).

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ABBREVIATIONS: ACh, acetylcholine; GTPγS, guanosine-5′-*O*-(3-thio)triphosphate; CHO, Chinese hamster ovary.

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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, α (Fig. 1). In this figure, positive cooperativity is present if $\alpha > 1$. The value of α 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 Birdsall, 1995; Jakubik et al., 1997).

In the case of gallamine, all reported α values are <1. Subsequently, alcuronium (Tucek et al., 1990) and strychnine (Lazareno and Birdsall, 1995; Proska and Tucek, 1995) have been shown to exhibit positive cooperativity with the antagonist radioligand [^{3}H]*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-[^{35}S]thio)triphosphate ([^{35}S]GTPγS) was from DuPont/NEN (Hounslow, Middlesex, UK). [γ-^{32}P]GTP was from Amersham International (Cardiff, Wales). Saponin, GDP, brucine sulfate, ACh chloride, Fura 2-acetoxymethyl ester, 5,5′-dithiobis(2-nitrobenzoic acid), 9-amino-1,2,3,4-tetrahydroacridine (tacrine), 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-Novabiochem (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_1–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 Birdsall, 1998).] The cells were grown in α-minimal essential medium (GIBCO, Grand Island, NY) containing 10% (v/v) newborn calf serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 2 mM glutamine at 37° 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 (40,000*g*, 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.

GTPγS 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 μg/ml. To the membrane suspension on ice was added the appropriate concentration of GDP followed by [^{35}S]GTPγS (final concentration 100 pM). 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_3 receptors and 10^{-6} M for M_2 and M_4 receptors. In some assays, saponin (10 μg/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 μg/ml. To the membrane suspension was added [γ-^{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 20 mM orthophosphoric acid plus 1 mg/ml BSA. After centrifugation (14,000*g*, 5 min), an aliquot of the supernatant, containing the released labeled phosphate, was counted for radioactivity. Assays were conducted in duplicate.

cAMP Assay. CHO cells expressing M_1 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 $MgSO_4$, 1.0 mM $NaHPO_4$, 5.6 mM

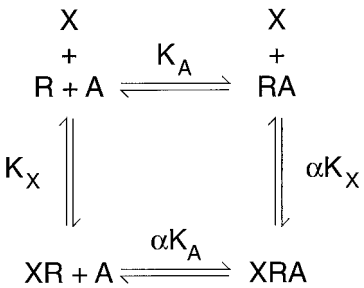

Fig. 1. Ternary complex allosteric model of the interaction of a ligand A with an allosteric agent X at a receptor R.

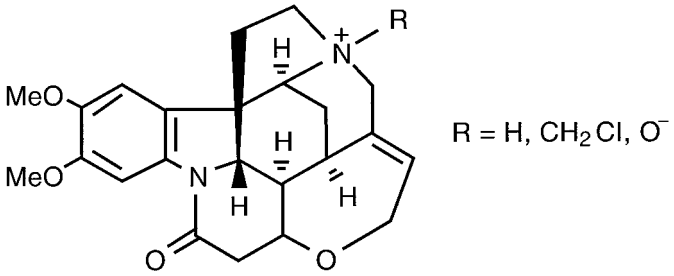

Fig. 2. Formulas of brucine (R = H, shown here as the protonated species), *N*-chloromethyl brucine (R = CH_2Cl), and brucine *N*-oxide (R = O^-).

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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 5×10^7 cells/ml. Aliquots of the cell suspension (0.1 ml) were incubated with various concentrations of ACh with or without brucine (100 μM) 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 μM) 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 excitement 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 μM 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 a 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-

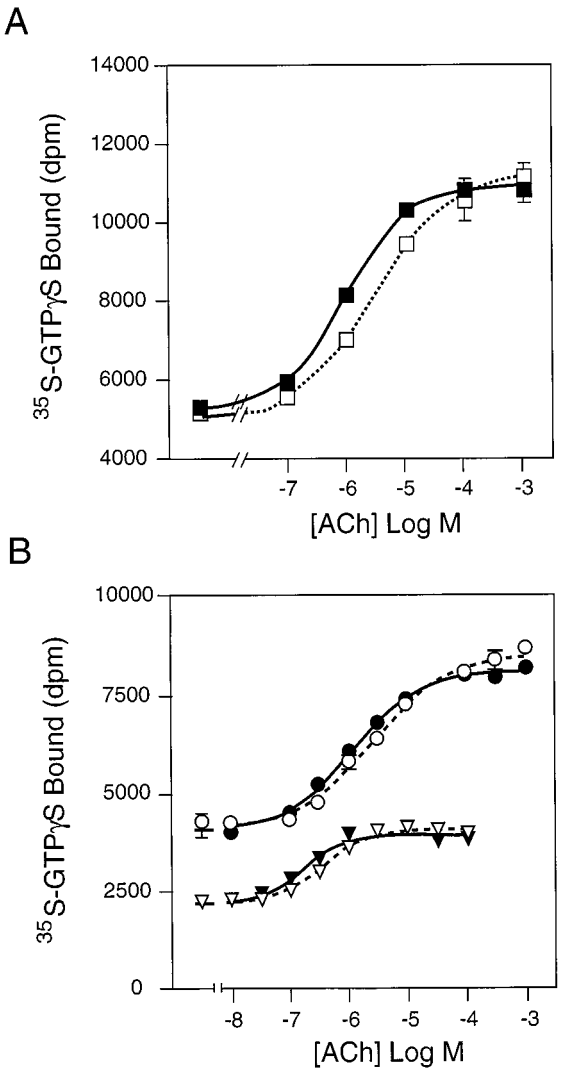

Fig. 3. Enhancement by brucine of ACh potency at M_1 receptors in assays of function in membranes. A, brucine (100 μM) increased the potency of ACh to stimulate [^{35}S]GTPγS to G proteins in M_1-CHO cell membranes. In this experiment, the EC_{50} value for ACh decreased from 2.8 μM (□) to 0.9 μM (■) without significantly affecting the basal response or maximal stimulation. B, enhancement of ACh-stimulated [^{35}S]GTPγS 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).

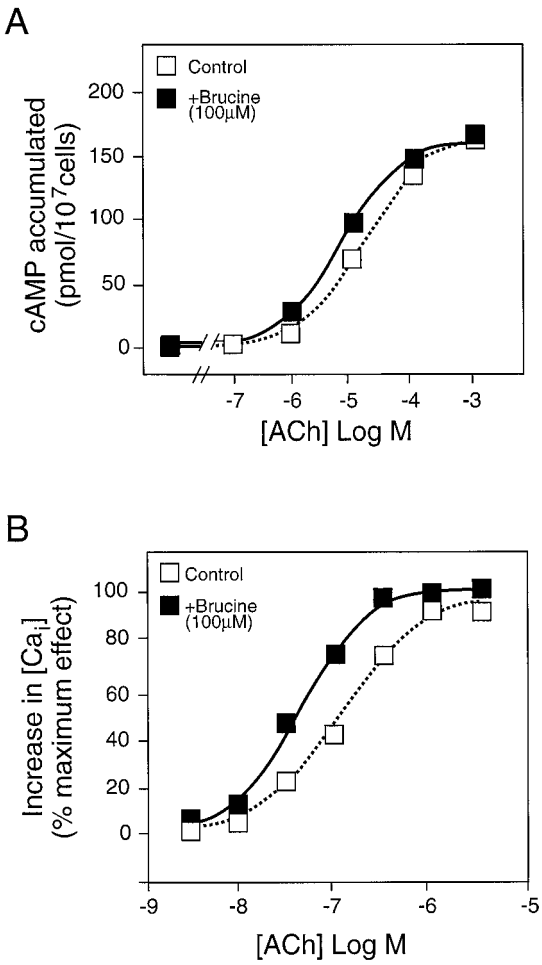

Fig. 4. Dose-response curves for the potentiation by brucine of ACh whole-cell M_1 muscarinic receptor responses. A, brucine (10^{-4} M) enhanced the potency of ACh to increase cAMP accumulation in M_1-CHO cells by 2.6-fold. B, dose-response curves generated from the data shown in Fig. 5 and additional data from the same experiment, normalized to the response to 10^{-6} M ACh alone, show that brucine (100 μM) produced a 3.0-fold increase in ACh potency.

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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°. 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_1 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_1 receptors (Lazareno et al., 1998). The positive cooperativity at M_1 receptors has been confirmed in [^{35}S]GTPγS functional assays in membranes where the ability of an agonist (in these experiments, ACh) to increase the rate of binding of [^{35}S]GTPγS 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 [^{35}S]GTPγS with an EC_{50} value of 2.8 μM. In the presence of brucine (10^{-4} M), the ACh potency increased 3-fold to 0.9 μM. As illustrated here, neither the basal level of [^{35}S]GTPγS 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.,

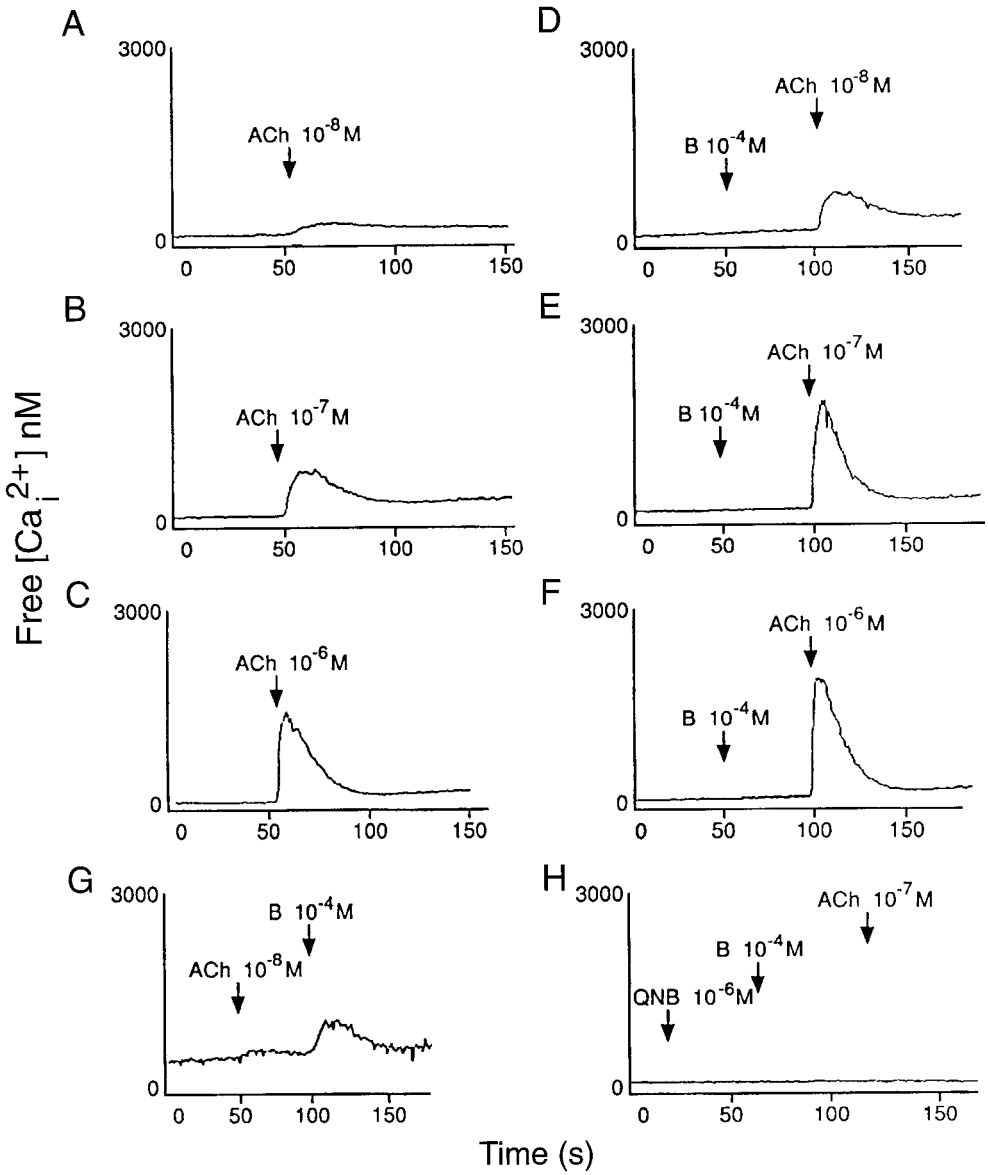

Fig. 5. Allosteric enhancement by brucine of a whole-cell M_1 Ca^{2+} response to ACh. ACh (10^{-8} to 10^{-6} M) produced a dose-dependent increase in intracellular Ca^{2+} concentration levels in M_1-CHO cells (A–C). The responses to 10^{-8} and 10^{-7} M ACh are significantly potentiated by 10^{-4} M brucine (D and E), whereas a much smaller effect on the response to 10^{-6} 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^{2+} 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.

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1995) and in some experiments with brucine (10^{-4} M and higher concentrations) that the basal and maximal levels of binding were affected. This can have the apparent effect of increasing ACh potency: the results of such experiments were not included in the data analysis. The mean enhancement of ACh potency by 10^{-4} M brucine in experiments where basal and maximal levels of binding were unaffected was 2.1 ± 0.4-fold (n = 4).

We have observed elsewhere that pertussis toxin treatment of cells generates membranes in which basal activity in GTPase and [^{35}S]GTPγS assays of function is reduced and the potency of ACh is increased up to 10-fold (Lazareno et al., 1993, Lazareno, 1997). Although a reduction in basal activity was not observed with the brucine analogs, it was considered that there was a slight chance that the observed enhancement of ACh potency by brucine is not an allosteric effect but an artifact mediated by an effect on $G_{i/o}$ proteins or a change in the G protein-coupling selectivity of M_1 receptors. [^{35}S]GTPγS assays were therefore performed on membranes of M_1-CHO cells treated with pertussis toxin (30 ng/ml) for 18 h before harvesting the membranes. These conditions were known from preliminary experiments to block essentially all $G_{i/o}$ protein function. These [^{35}S]GTPγS 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^{-4} M brucine without significantly affecting the slope. The effect of pertussis toxin treatment was to reduce basal binding ≈50%, 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 ± 0.1-fold, n = 2) was retained in the pertussis toxin-treated membranes, indicating that brucine equally enhances the ability of ACh-M_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_1 receptor-linked whole-cell responses. At a concentration of 10^{-4} M, it potentiated the EC_{50} value of ACh-stimulated cAMP accumulation in M_1-CHO cells by 2.4 ± 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^{2+} concentration in the same M_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 ± 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^{2+} signaling response to submaximal concentrations of ACh (10^{-8} and 10^{-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^{-6} M) (Fig. 5H).

Allosteric Enhancement of M_4 Receptor Function in Membranes by Brucine *N*-Oxide. An analog of brucine, brucine *N*-oxide, exhibits a 1.4 ± 0.1-fold positive cooperativity with ACh in binding studies on M_4 receptors. (Lazareno et al., 1998). This is mirrored in functional studies by a 2.8 ± 0.1-fold (n = 3) increase in ACh potency at M_4 receptors by 10^{-3} M brucine *N*-oxide in a GTPase assay of function (Fig. 6). In measures of ACh-stimulated GTPγ^{35}S binding at M_4 receptors, the EC_{50} value for ACh is about 2 to 3 × 10^{-7} M (Lazareno et al., 1993; Lazareno and Birdsall, 1993); significant enhancements of the actions of a submaximal single concentration ACh (10^{-7} M) were observed using 3 × 10^{-4} or 10^{-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 × 10^{-4} M brucine *N*-oxide increased ACh potency at M_4 receptors by 1.9 ± 0.3-fold (n = 2, data not shown).

In binding studies, brucine *N*-oxide is positively cooperative with ACh at M_3 receptors, neutral at M_1 receptors, and weakly negatively cooperative at M_2 receptors (Lazareno et al., 1998). This qualitative profile was also observed in [^{35}S]GTPγS functional studies on these subtypes (data not shown).

Allosteric Enhancement of M_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_3 receptors but not at the other subtypes, being very slightly negatively cooperative at M_1, strongly negative at M_2 receptors, and essentially neutrally cooperative at M_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 [^{35}S]GTPγS binding to membranes of M_1–M_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_1 and M_4 receptors were observed (range of pEC_{50} val-

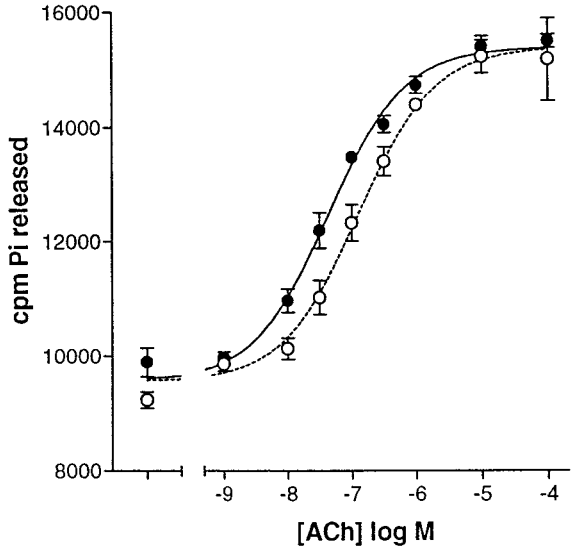

Fig. 6. Enhancement by brucine *N*-oxide of ACh-stimulated GTPase activity at M_4 receptors. In the simultaneous analysis of the data, the basal and maximal values of the ACh dose-response curves, as well as the Hill slopes (0.74), were constrained to be equal in the presence (●) or absence (○) of brucine *N*-oxide (10^{-3} M). The enhancement of ACh potency was 2.9-fold in this experiment.

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ues, 5.80–5.97 and 6.80–6.97 at M_1 and M_4 receptors, respectively). The results at M_2 and M_3 receptors illustrate clearly the qualitatively different effects of chloromethylbrucine at these subtypes. Increasing concentrations of *N*-chloromethylbrucine progressively shift the ACh dose-respose curve for M_2 receptors to the right of the control (dashed) curve, whereas the M_3 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 M_2 and M_3 receptors, respectively. There is a good agreement between the affinities of *N*-chloromethyl brucine and its cooperativities with ACh at M_2 and M_3 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 M_3 whole tissue preparation, the isolated guinea pig ileum. *N*-Chloromethyl brucine (20–200 μM) produced a 1.8- to 2.6-fold increase in the submaximal contractions produced by a low concentration (2×10^{-9} M) of ACh ($n = 4$, data not shown) but had no effect on the maximal contractions generated using higher concentrations of ACh (2×10^{-7} to 2×10^{-6} M). It was also possible to observe a dose-dependent potentiation by *N*-chloromethyl brucine (2–200 μM) of the electrically stimulated contraction of isolated strips of guinea pig ileum, a whole tissue response mediated via ACh release and M_3 receptor activation (Fig. 8). The threshold concentration of *N*-chloromethyl brucine for the potentiation of the submaximal stimuli was 2 to 10 μM and a more than 3-fold enhancement was observed at 200 μM. 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 μM) 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} 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 μM). *N*-Chloromethyl brucine (2–200 μM) 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 M_2 autoreceptor rather than a postsynaptic enhancement of M_3

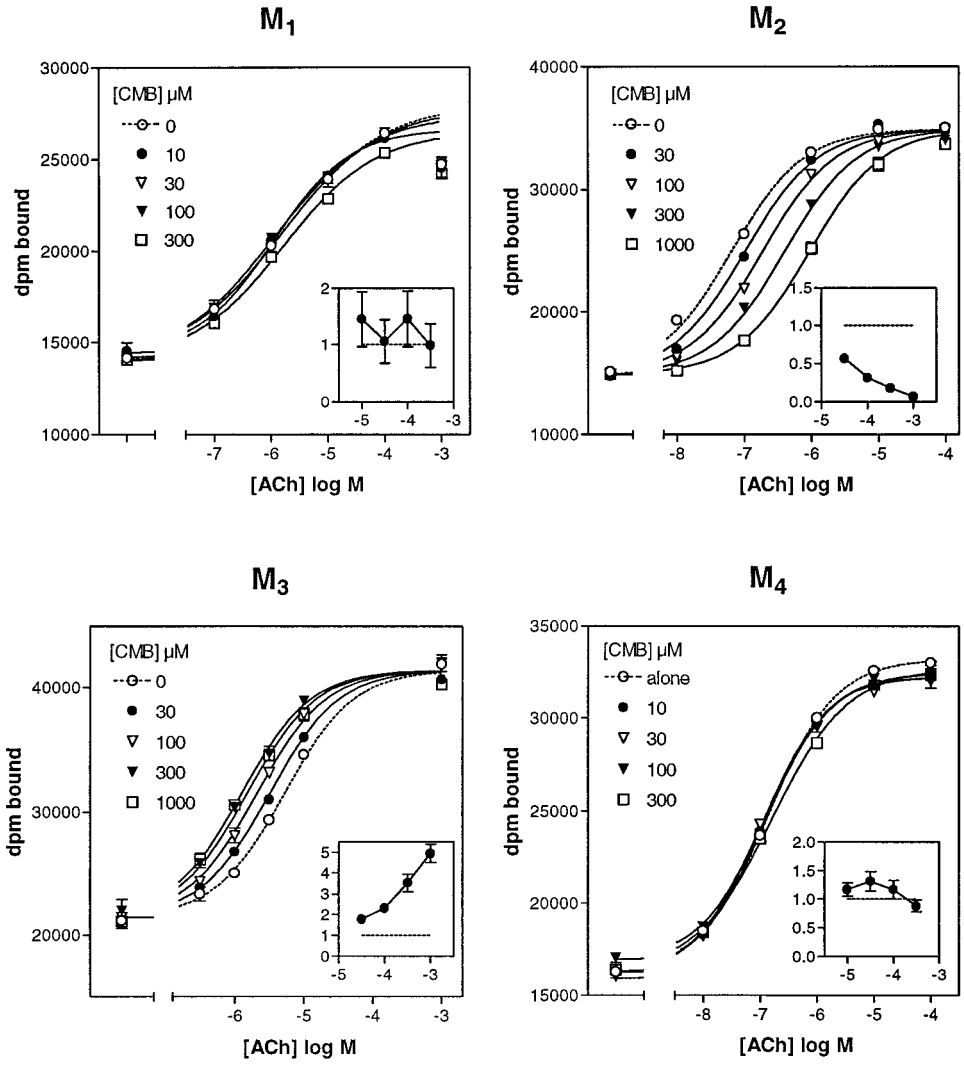

Fig. 7. Allosteric modulation by *N*-chloromethyl brucine of ACh-stimulated [^{35}S]GTPγS binding at M_1–M_4 receptors. At M_1 and M_4 receptors, there was no dose-dependent change in the pEC_{50} value of ACh within the experiment illustrated here, and in additional experiments, the mean pEC_{50} values at the two subtypes was 5.87 ± 0.09 and 6.90 ± 0.09 (mean ± range/2, $n = 5$). At M_2 and M_3 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 M_2 and M_3 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 M_2 and M_3 receptors, 10 μg/ml saponin was present in the assays. *Insets*, fold changes in the EC_{50} values as a function of log[*N*-chloromethyl brucine].

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receptors. However, in contrast to *N*-chloromethyl brucine, the M_2-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_3 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_1, M_3, and M_4 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, 6, 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 gallamine 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 [^{3}H]*N*-methylscopolamine such that occupancy of the allosteric site precludes access or egress of the antagonist from the binding site. However, we find no evidence that under our experimental conditions, ACh access to its binding site is blocked by these agents; this would result in the brucine analogs slowing the both the onset and offset of the ACh responses. Our results are in accord with the less pronounced maximal slowing of the dissociation kinetics of [^{3}H]ACh from M_2 receptors by gallamine, alcuronium (Gnagey and Ellis, 1996), and brucine analogs (S. Lazareno and N.J.M. Birdsall, unpublished results).

The concentrations of the brucines that produce their effects on function and the directions and magnitudes of the allosteric effects are both qualitatively and quantitatively compatible with the predictions from the results of binding studies and are independent of the nature of the response being measured. This suggests that the brucine analogs increase only the affinity of ACh and that all the brucine analog-receptor-ACh ternary complexes have the same ability ("efficacy") as the binary ACh-receptor complex to activate their effector mechanisms. Even when the muscarinic receptor can couple to multiple G proteins, as in the case of the M_1 receptor (Lazareno et al., 1993, Offermans et al., 1994), the experiments involving pertussis toxin treatment of the cells demonstrate that the actions of brucine at this receptor subtype do not alter the selective ability of ACh to activate pertussis toxin-insensitive and -sensitive G proteins (Fig. 3, A and B).

Because the observed positive cooperativities with these compounds are relatively small, it was generally difficult

TABLE 1

Comparison of the parameters for the allosteric interaction of *N*-chloromethyl brucine with acetylcholine at M_2 and M_3 receptors measured in binding and functional studies (n = 5).

Subtype	Binding Studies[a]		Function	
	Log Affinity	Cooperativity with ACh	Log Affinity	Cooperativity with ACh
	log K_X	α	*log* K_X	α
M_2	4.22 ± 0.11	0.09 ± 0.02	4.30 ± 0.10	0.02 ± 0.01
M_3	3.66 ± 0.13	3.3 ± 0.2	3.84 ± 0.02	3.8 ± 0.5

[a] The binding data are from Lazareno et al. (1998).

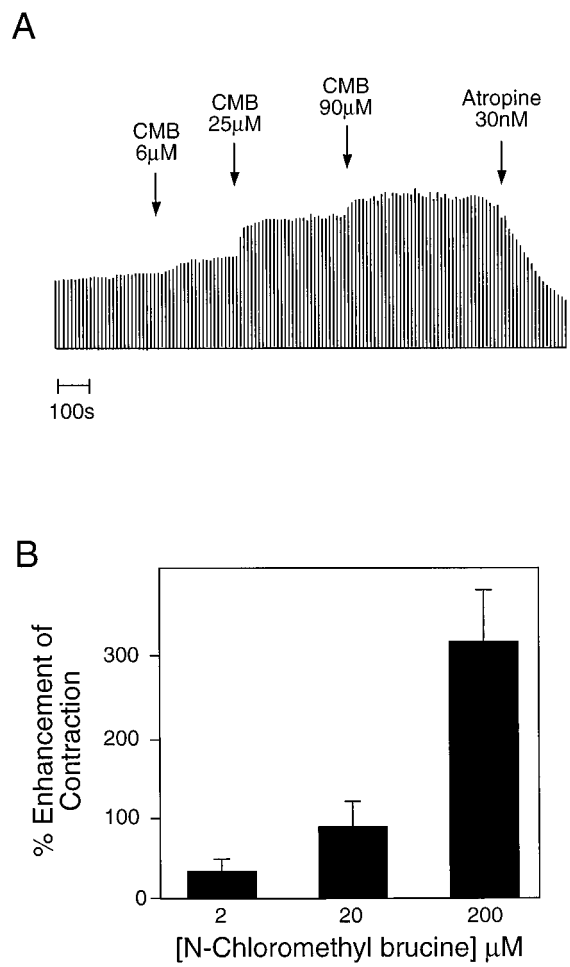

Fig. 8. A, *N*-Chloromethyl brucine, in a dose-dependent manner, enhanced the field-stimulated contractions of isolated guinea pig ileum strips. The contractions were inhibited by atropine (30 nM). B, histogram of the percentage enhancement of contraction produced in four independent experiments of the type illustrated in A. Data are expressed as mean ± S.E.M.

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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_X and α. In the case of *N*-chloromethyl brucine, however, it was possible to demonstrate clearly that its positive and negative allosteric effects on ACh -stimulated GTPγ^{35}S binding, mediated via M_3 and M_2 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 (α = 3–4) and inhibitor (α = 0.02–0.09), a ratio of about 60, at 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 μM *N*-chloromethyl brucine on ACh function at M_1 and M_4 receptors. This is true despite the fact that 10 to 300 μM *N*-chloromethyl brucine has dramatic slowing effects on the dissociation rate constant of [^{3}H]*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 (α = 1.0 ± 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_3 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 nicotinic ACh receptors, presynaptic M_2 inhibitory autoreceptors, or acetylcholinesterase 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 [^{3}H]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].

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