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The effects of brucine and alcuronium on the inhibition of [^{3}H]acetylcholine release from rat striatum by muscarinic receptor agonists

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1 Radioligand binding experiments indicate that the affinity of muscarinic receptors for their agonists may be enhanced by allosteric modulators. We have now investigated if brucine can enhance the inhibitory effects of muscarinic receptor agonists on the electrically evoked release of [^{3}H]acetylcholine ([^{3}H]ACh) from superfused slices of rat striatum.

2 The evoked release of [^{3}H]ACh was inhibited by all agonists tested (i.e., furmethide, oxotremorine-M, bethanechol and oxotremorine).

3 Brucine enhanced the inhibitory effects of furmethide, oxotremorine-M and bethanechol on the evoked [^{3}H]ACh release without altering the inhibitory effect of oxotremorine.

4 Alcuronium was applied for comparison and found to diminish the inhibitory effect of furmethide on the evoked [^{3}H]ACh release.

5 The results demonstrate that it is possible both to enhance and diminish the functional effects of muscarinic receptor agonists by allosteric modulators.

6 The direction of the observed effects of brucine and alcuronium on [^{3}H]ACh release fully agrees with the effects of these modulators on the affinities of human M_4 receptors for furmethide, oxotremorine-M, bethanechol and oxotremorine, as described by Jakubík *et al*. (1997). This supports the view that the presynaptic muscarinic receptors responsible for the autoinhibition of ACh release in rat striatum belong to the M_4 muscarinic receptor subtype.

Keywords: Acetylcholine release; presynaptic autoreceptors; muscarinic receptors; allosteric modulation; cholinergic neurons; striatum; brucine; alcuronium

Introduction

It has been known for some time that the affinity of muscarinic receptors for their agonists and antagonists may be allosterically diminished (Clark & Mitchelson, 1976; Stockton *et al*., 1983; review Lee & El-Fakahany, 1991). More recently, it has been discovered that the affinity of muscarinic receptors for their antagonists may be also enhanced by allosteric modulators; this was shown in radioligand binding experiments on isolated membranes (Tuček *et al*., 1990; Proška & Tuček, 1994; 1995; Jakubík & Tuček, 1994a,b; Lazareno & Birdsall, 1995; Guo *et al*., 1995), intact cells (Jakubík *et al*., 1995) and solubilized receptors (Musílková & Tuček, 1995). Recently, it has been found in experiments with radioligand binding that the allosteric modulators may enhance the affinity of muscarinic receptors also for their agonists (Birdsall *et al*., 1997; Jakubík *et al*., 1997).

The interactions between the classical ligands and the allosteric modulators vary in their direction and strength, depending on the subtype of the muscarinic receptor involved and the chemical nature of either ligand (Tuček & Proška, 1995; Birdsall *et al*., 1997). For example, the affinities for furmethide, oxotremorine-M and bethanechol were enhanced by brucine on human M_4 muscarinic receptors but diminished on human M_2 muscarinic receptors (Table 1). The affinity for oxotremorine was strongly diminished by brucine on the M_2 muscarinic receptor subtype but very little changed on the M_4 muscarinic receptor subtype, and the affinity for furmethide was strongly diminished by alcuronium both on the M_2 and the M_4 receptor subtypes (Jakubík *et al*., 1997).

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The discovery of the positive effects of allosteric modulators on the affinities of muscarinic receptors for their agonists in experiments with radioligand binding raises the important question of whether the positive action of the allosteric modulators may be also revealed in investigations of agonist functional effects. The possibility of allosterically enhancing the functional effects of muscarinic receptor agonists might have a profound impact on the pharmacology of muscarinic neurotransmission.

It is well known that muscarinic receptor antagonists facilitate and muscarinic receptor agonists inhibit the release of acetylcholine (ACh) from cholinergic nerve terminals and that their action depends on the function of muscarinic autoreceptors located on terminal branches of cholinergic neurons (Molenaar & Polak, 1980; Illes, 1986; Doležal *et al*., 1989; Doležal & Tuček, 1993). Striatal slices were frequently used in investigations of the control of ACh release by muscarinic autoreceptors (James & Cubeddu, 1984; Doležal & Wecker, 1990). In the present work, we asked the question whether the inhibitory effect of muscarinic receptor agonists on the release of ACh from superfused striatal slices may be specifically enhanced by an allosteric modulator.

Methods

Preparation and superfusion of striatal slices

Male Wistar rats aged 2–3 months were sacrificed by cervical dislocation and decapitation. Coronal striatal slices were prepared using McIlwain's tissue chopper set at 0.35 mm slice

thickness. They were washed in an aqueous superfusion medium containing (mM) NaCl, 123; KCl,3; $CaCl_2$, 1.3; $MgSO_4$ 1; NaH_2PO_4, 1.2; $NaHCO_3$ 25 and glucose 10. The medium was saturated with 95% O_2 and 5% CO_2 and its pH was 7.4. Eight to ten slices were loaded with [^{3}H]choline (19–32 nmol/l; 78 Ci/mmol) during a preincubation lasting 30 min at 37°C. They were then washed and randomly transferred into the superfusion chambers, one slice per chamber. In the chambers, they were superfused (0.5 ml/min, 37°C) for 1 h with the superfusion medium including hemicholinium-3 (10 μM), and the superfusate was discarded. After 1 h, the superfusate was collected every 4 min and the fractions were designated as F1 (0–4 min), F2 (4–8 min) etc. until F12 (44–48 min).

Measurement of stimulation-evoked ACh release

The slices were stimulated electrically twice for 20 s, at the beginning of the third and ninth collection period (i.e., 8 and 32 min after the superfusate started to be collected). The stimulation was by 60 square pulses lasting 2 ms at a frequency of 3 Hz, with a voltage gradient of 10 V across the inter-electrode distance of 5 mm, yielding a current of 35 mA. This strength of stimulus was previously found to be effective in a similar superfusion system (Doležal *et al.*, 1992) and the ACh released during the short stimulation under superfusion did not produce autoinhibition of ACh release, as can be judged from the observation that the evoked release was not enhanced by atropine (data not shown).

Unless indicated otherwise, the allosteric modulator was added to the medium 30 min before the start of the first collection period and was present throughout the experiment. The first stimulation was always performed in the absence of muscarinic receptor agonists. The agonists were added to the medium starting from the beginning of the 6th collection period (12 min after the start of the first stimulation, 12 min before the start of the second stimulation) and remained present until the end of the 12th collection period. After the end of the 12th collection period, the slices were removed from the superfusion chamber and dissolved in 1 ml of 100 mM NaOH. The amounts of radioactivity present in individual 4 min fractions of the superfusate and in the slices were determined by scintillation counting.

The stimulated release of radioactivity was computed as the difference between the radioactivity in (F3 + F4) or (F9 + F10) and the radioactivity in (F2 + F5) or (F8 + F11), respectively. It was expressed as the fractional release, i.e. as the percentage of the total radioactivity present in the slice at the beginning of the first or the second stimulation. This was performed as described in detail by Doležal *et al.* (1992). It was assumed that the stimulated release of ^{3}H is an adequate measure of the release of [^{3}H]ACh (Richardson & Szerb, 1974; Doležal *et al.*, 1992). The effect of muscarinic receptor agonists on the release was determined by computing the S2/S1 ratios, i.e. the ratios of [fractional [^{3}H]ACh release evoked by the second stimulation]/[fractional [^{3}H]ACh release evoked by the first stimulation]. The action of allosteric modulators was evaluated by comparing the effects of muscarinic receptor agonists on [^{3}H]ACh release revealed in experiments performed either with or without the modulators.

Source of reagents

[^{3}H]Choline was from Amersham Int. (Amersham, England), bethanechol, brucine, oxotremorine, oxotremorine-M, tetrodotoxin and choline oxidase were from Sigma Chemical (St. Louis, MO), furmethide was synthesized by Ing. Bielavský (Hradec Králové) and alcuronium was kindly provided by Hoffmann-LaRoche (Basel, Switzerland).

Results

Evoked [^{3}H]ACh release in the absence of muscarinic receptor agonists and in the presence of brucine or alcuronium

Data on the release of ^{3}H obtained in superfusion experiments in the absence of added muscarinic receptor ligands have been indicated by full symbols in Figure 1. Open symbols describe the release of ^{3}H from the slices incubated, starting from the sixth collection period, with 100 μM brucine (top) or with 25 μM alcuronium (bottom). The concentrations of brucine and alcuronium were chosen so as to be at least 4-fold higher than the concentrations which had been found to ensure 50% occupancy of the allosteric binding sites on human M_2 and M_4 muscarinic receptor subtypes (Jakubík *et al.*, 1997; Table 1),

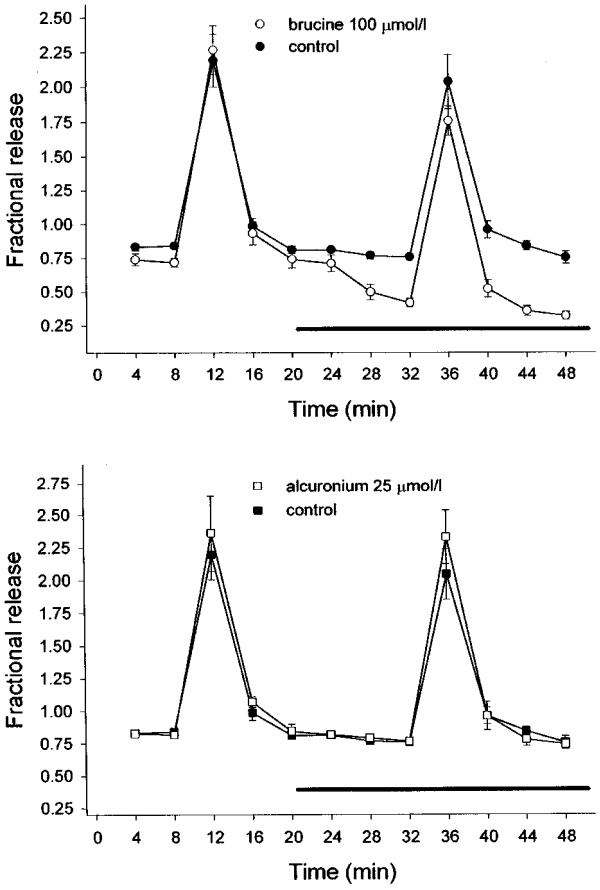

Figure 1 [^{3}H]ACh release from superfused striatal slices at rest and during stimulation in the absence or presence of brucine (top) and alcuronium (bottom). The superfusion fluid was collected every 4 min. Electric stimulation was applied at the beginning of the third and ninth collection periods. Brucine (100 μM; top) or alcuronium (25 μM; bottom) were added to the superfusion fluid starting from the sixth collection period and stayed till the end of the superfusion, as indicated by the black horizontal bar. Abscissa: Time after the start of the collection (min). Ordinate: The radioactivity released during individual 4 min collection periods, expressed as per cent of total radioactivity present in the slice at the beginning of this collection period. Full symbols: Control samples. Open circles: Samples with brucine. Open squares: Samples with alcuronium. Each point represents mean ± s.e.mean of data obtained on six slices in the case of brucine and on three slices in the case of alcuronium.

Table 1 Changes of K_d values for the binding of selected agonists by the M_2 and M_4 subtypes of muscarinic receptors, induced by the allosteric modulators brucine and alcuronium

Allosteric modulator	*Agonist*	*Fold change of K_d for the agonist induced by the modulator on the M_2 receptor subtype*	*Fold change of K_d for the agonist induced by the modulator on the M_4 receptor subtype*
Brucine	Furmethide	71	0.23
Brucine	Oxotremorine-M	3.3	0.24
Brucine	Bethanechol	4.9	0.22
Brucine	Oxotremorine	5.1	1.39
Alcuronium	Furmethide	8.4	9.3

Data are from Jakubík *et al.* (1997) and originate from radioligand binding experiments on CHO cell lines expressing human genes for individual subtypes of muscarinic receptors. The indicated changes of K_d values occur at saturating concentrations of the allosteric modulators. The affinities for brucine corresponded to K_d values of 24.3 μM and 0.95 μM on the M_2 and $_4$ receptors, respectively, and those for alcuronium to 0.73 μM and 2.49 μM on the M_2 and M_4 receptors, respectively

which are the subtypes most likely to participate in the control of ACh release. Neither brucine nor alcuronium changed the release of ^{3}H evoked by electric stimulation. In experiments with brucine, the S2/S1 ratio (mean $\pm$ s.e.mean) was 0.923 ± 0.155 ($n=6$) in control samples and 0.897 ± 0.158 ($n=6$) in samples exposed to the modulator during the second stimulation. In experiments with alcuronium, the S2/S1 ratio was 0.942 ± 0.022 ($n=3$) in control slices and 0.977 ± 0.038 ($n=3$) in slices exposed to the modulator.

Effect of brucine on the spontaneous efflux of ^{3}H

As can be seen from Figure 1, the spontaneous efflux of ^{3}H was diminished by brucine. We performed two sets of control experiments to clarify the meaning of this phenomenon:

(1) The effect of 100 μM brucine on the spontaneous ^{3}H efflux was not diminished by 1 μM tetrodotoxin (Figure 2), although this concentration of tetrodotoxin completely prevented the electrically evoked ^{3}H release in the present experiments (data not shown) and had been found to block the release of ACh induced by endogenous neural activity in tissues during *in vitro* incubations in previous work (Doležal & Tuček, 1983; 1992).

(2) Experiments were performed on slices pretreated with paraoxon (50 μM), an irreversible inhibitor of cholinesterases. This permitted the measurement of released [^{3}H]ACh, which was extracted from the medium by means of sodium tetraphenylboron dissolved in butyronitrile (Fonnum, 1969), while the accompanying [^{3}H]choline was destroyed by choline oxidase. During an incubation period of 8 min, the efflux of ^{3}H represented 2.42 ± 0.08% of total radioactivity in control slices and 1.57 ± 0.09% in slices incubated with 100 μM brucine. The release of [^{3}H]ACh represented 1.10 ± 0.04% of total radioactivity in control slices and 1.02 ± 0.03% in slices incubated with 100 μM brucine. These data are means $\pm$ s.e.mean of 8 determinations. The effect of brucine was highly significant with regard to the total efflux of radioactivity but insignificant with regard to the efflux of [^{3}H]ACh.

We concluded from these two types of experiments that the effect of brucine on the spontaneous efflux of ^{3}H was not related to the endogenous (spontaneous) electrical activity of neurons and nerve fibres in the slices and was not due to a diminished release of [^{3}H]ACh from the tissue.

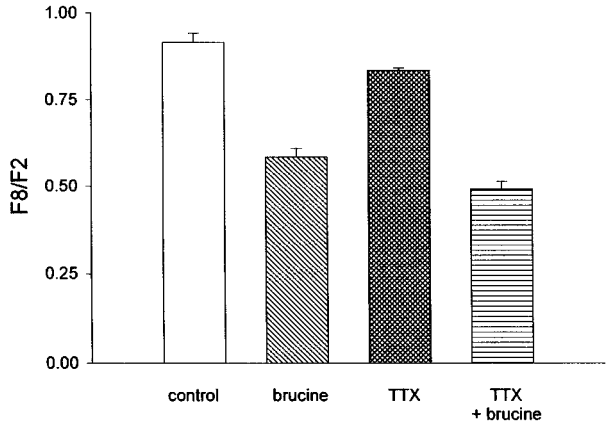

Figure 2 Effects of brucine (100 μM) and tetrodotoxin (TTX, 1 μM) on the spontaneous efflux of ^{3}H from striatal slices. The experiments were arranged similarly as in Figure 1a, and either brucine alone, or tetrodotoxin alone, or both brucine and tetrodotoxin were present in the superfusate starting from the 21st minute of perfusion (i.e., from the start of the sixth collection period). The spontaneous effluxes of ^{3}H occurring during the eighth collection period (F8, immediately preceding the second stimulation) and the second collection period (F2, immediately preceding the first stimulation) were compared by computing the F8/F2 ratios (expressed in terms of fractional release), which are shown on the graph (mean $\pm$ s.e.mean, $n=3-9$). The F8/F2 ratios for brucine alone and brucine plus tetrodotoxin were significantly different ($P<0.05$) from those for controls or tetrodotoxin alone (ANOVA and Bonferroni's multiple comparison test).

[^{3}H]ACh release in the presence of furmethide and brucine or alcuronium

When furmethide was added to the superfusion medium 12 min before the second stimulation, the release of ^{3}H induced by the second stimulation was progressively diminished at increasing concentrations of the agonist (circles in Figure 3a). The inhibitory effect of furmethide on the release of ^{3}H was considerably enhanced by 100 μM brucine (triangles in Figure 3a). On the other hand, it was considerably diminished in the presence of 25 μM alcuronium (squares in Figure 3a).

[^{3}H]ACh release in the presence of oxotremorine-M, bethanechol, or oxotremorine, with and without brucine

Oxotremorine-M (Figure 3b), bethanechol (Figure 3c) and oxotremorine (Figure 3d) all inhibited the electrically evoked release of ^{3}H. It may be seen from Figure 3b that the inhibition of ^{3}H release induced by 30–300 nM oxotremorine-M was more potent in the presence of brucine than in its absence, while the maximum inhibition induced by 1000 nM oxotremorine-M was the same in the absence and the presence of the allosteric modulator. Brucine caused a leftward shift of the concentration-response curve in experiments with bethanechol

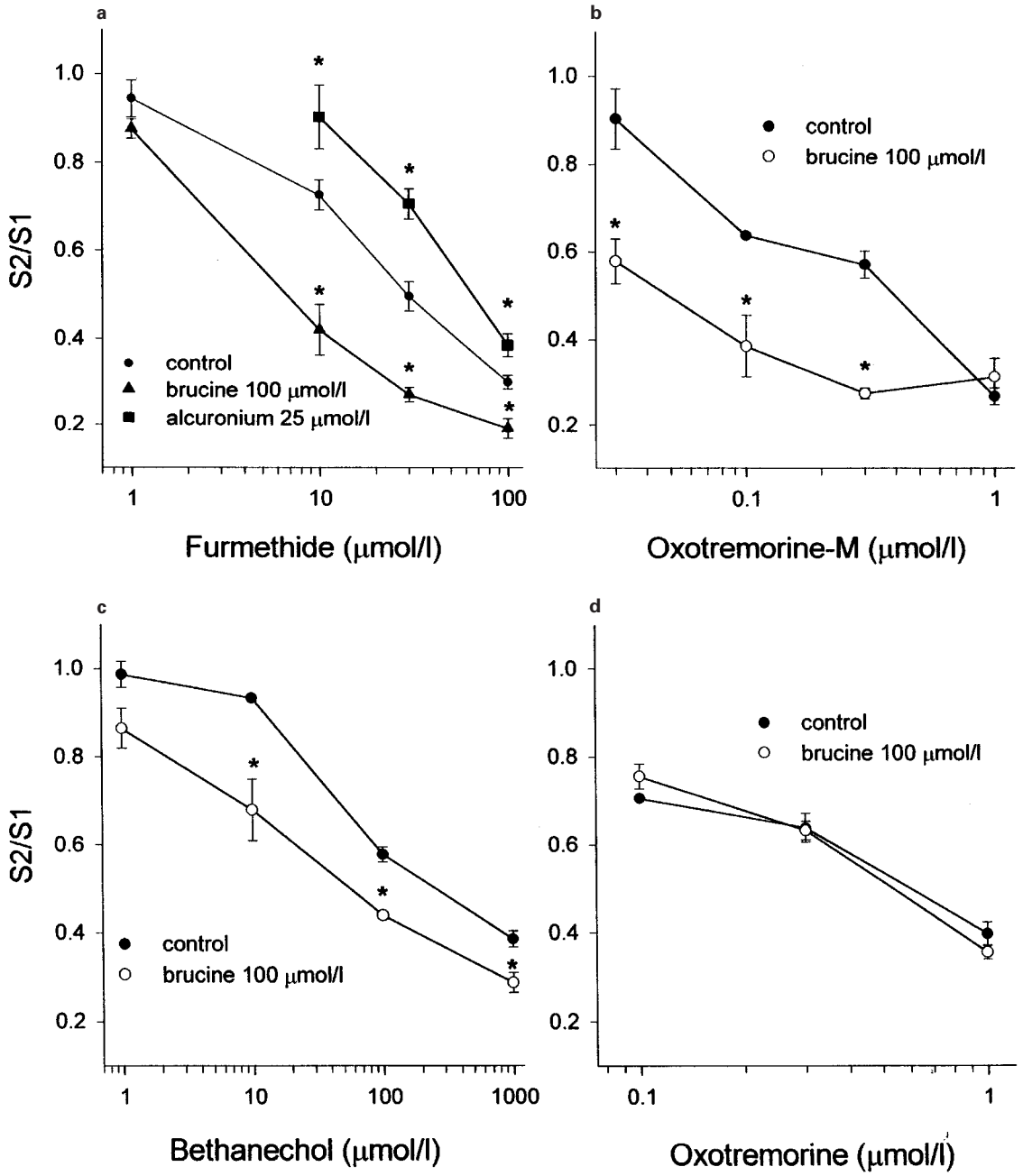

Figure 3 Concentration-response curves for the effects of (a) furmethide alone (circles), furmethide in the presence of 100 μM brucine (triangles) and furmethide in the presence of 25 μM alcuronium (squares), (b) oxotremorine-M alone (full circles), and oxotremorine-M in the presence of 100 μM brucine (empty circles), (c) bethanechol alone (full circles), and bethanechol in the presence of 100 μM brucine (empty circles) and (d) oxotremorine alone (full circles), and oxotremorine in the presence of 100 μM brucine (empty circles), on the release of [^{3}H]ACh from striatal circles. The S2/S1 paradigm was applied as described in Methods. Each point represents the mean ± s.e.mean of data obtained on 3–6 slices. Abscissa: Concentration of the agonist. Ordinate: S2/S1 ratio. *, significantly different ($P<0.05$) from samples exposed to the agonist alone, without the allosteric modulator (Student's two-tailed t-test).

(Figure 3c), but it had no effect on the course of the concentration-response curve in experiments with oxotremorine (Figure 3d).

Discussion

The inhibitory action of muscarinic receptors on the release of ACh from striatal cholinergic neurons is well known (James & Cubeddu, 1984; Drukarch *et al.*, 1990; Doležal & Wecker, 1990; Doležal & Tuček, 1990). Available evidence suggests that muscarinic receptors responsible for the inhibition belong either to the M_2 or the M_4 subtype, or to both (Hersch *et al.*, 1994; Billard *et al.*, 1995; Yan & Surmeier, 1996; see also Miller *et al.*, 1992; Vanucchi & Pepeu, 1995, for the hippocampus). However, the prevalence of the M_4 mRNA (Buckley *et al.*, 1988; Wei *et al.*, 1994) and M_4 protein (Yasuda *et al.*, 1993) is a characteristic feature of rat striatum. Data from a recent quantitative immunocytochemical study indicate that, of the total population of striatal muscarinic receptors in adult rats, 66% belong to the M_4, 23% to the M_1, 9% to the M_2 and 3% to the M_3 subtype (Tice *et al.*, 1996). Biochemically, the prevalence of the M_4 (compared to the M_2) receptor subtype in the striatum is supported by data indicating that it is the M_4

subtype which is responsible for the inhibition of adenylyl cyclase in striatal membranes by muscarinic receptor agonists (Onali *et al*., 1994; Olianas *et al*., 1996). Experiments with subtype-selective antimuscarinic toxins indicate that more than 90% of muscarinic binding sites in rat striatum belong to the M_4 and M_1 receptor subtypes (Purkerson & Potter, 1998).

Jakubík *et al*. (1997) performed experiments on muscarinic receptors expressed in Chinese hamster ovary (CHO) cell lines stably transfected with human genes for the M_1-M_4 receptor subtypes and determined the affinities of the receptors for 12 muscarinic agonists in the absence and presence of five allosteric modulators. The effect of individual allosteric modulators on the affinities for individual muscarinic receptor agonists varied between receptor subtypes. We have summarized in Table 1 those of the data which are most relevant to the present experiments. Although these data originate from a different biological material (i.e., from the CHO cells expressing human genes rather than from the rat brain), the amino acid composition, properties and allosteric regulatory mechanisms of muscarinic receptor subtypes are highly conserved in homoiothermic animals (Wess, 1995), and the binding properties of the human and the rat muscarinic receptors are virtually identical (Kovacs *et al*., 1998). It is therefore likely that data in Table 1 may serve as a sound basis for the interpretation of the results obtained on rat brain tissue.

Our data indicate that brucine enhances the inhibitory effects of furmethide, oxotremorin-M and bethanechol (but not of oxotremorine) on [^{3}H]ACh release. They are in excellent agreement with the data by Jakubík *et al*. (1997) on the positive cooperativity between the binding of brucine and furmethide, oxotremorine-M and bethanechol (but not oxotremorine) to the M_4 receptor subtype.

It might be suspected that, for some unknown reason, all allosteric modulators enhance the inhibitory effect of muscarinic agonists on [^{3}H]ACh release. The difference between the effects of brucine and alcuronium on the inhibition induced by furmethide (Figure 3a) indicates that this is not so. In agreement with the negative effect of alcuronium on the affinity of M_4 receptors for furmethide in work with radioligand binding (Jakubík *et al*., 1997), the inhibitory effect of furmethide on [^{3}H]ACh release was diminished by alcuronium in the present experiments.

The following main conclusions appear justified: (1) Brucine is able to increase the inhibitory effects of muscarinic receptor agonists furmethide, oxotremorine-M and bethanechol (but not those of oxotremorine) on the electrically evoked release of ACh in rat striatum, whereas the inhibitory effect of furmethide can be diminished by alcuronium; (2) The correlation between the effects of brucine on the inhibition of ACh release by individual agonists and on the affinities of M_4 receptors for these agonists suggests that the M_4 muscarinic receptor subtype is involved in the inhibition of ACh release by muscarinic receptor agonists in rat striatum.

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