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SHORT COMMUNICATIONS

Presynaptic Antagonist-Postsynaptic Agonist at Muscarinic Cholinergic Synapses

N-Methyl-*N*-(1-methyl-4-pyrrolidino-2-butynyl)acetamide

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SUMMARY

A muscarinic cholinergic ligand, *N*-methyl-N-(1-methyl-4-pyrrolidino-2-butynyl)-acetamide (Compound BM-5), was shown to act simultaneously as a presynaptic antagonist and as a postsynaptic agonist at peripheral and central muscarinic synapses. In the presence of eserine it enhanced release of [^{3}H]acetylcholine evoked by electrical stimulation in the guinea pig ileum longitudinal muscle myenteric plexus preparation. It also enhanced the release of [^{3}H]acetylcholine evoked by potassium (25 mM) from rat hippocampal slices. It contracted the ileum and stimulated the synthesis of cyclic guanosine 3′,5′-monophosphate in rat hippocampal slices and in human lymphocytes in a manner similar to the muscarinic agonist oxotremorine. This effect of BM-5 could be blocked by atropine.

The over-all action of a muscarinic agent at synapses in the peripheral and central nervous system depends on its efficacy and affinity not only at the classical (postsynaptic) muscarinic receptors (1, 2) but also on its interactions with the presynaptic autoreceptors which feedback-regulate the release of ACh[3] (2). The actions of the classical muscarinic antagonist atropine in the guinea pig ileum myenteric plexus or in cholinergic synapses in the rat hippocampus are 2-fold and partially opposing: (a) atropine enhances stimulus-evoked release of ACh by blocking the presynaptic autoreceptors, and (b) atropine blocks the postsynaptic actions of muscarinic agonists via its high-affinity binding to the postsynaptic receptors (2). Similarly, exogenous muscarinic agonists such as oxotremorine also have two targets. These agonists enhance the cholinergic transmission at the synapse, since they activate the postsynaptic receptors, but at the same time they decrease the evoked release of ACh, since they activate the presynaptic autoreceptors as well. Ideally, an agent that will unequivocally enhance cholinergic transmission ought to behave as a *presynaptic antagonist* and as a *postsynaptic agonist, simultaneously*. In this way the concentrations of both the exogenous and endogenous agonists may reach maximal levels in the synaptic cleft. We report here that an acetylenic compound related to oxotremorine appears to behave *in vitro*, in the presence of eserine, as a presynaptic antagonist and postsynaptic agonist in the central and peripheral nervous system and in non-nerve tissue.

Since 1961, when Cho *et al.* (3) synthesized oxotremorine, the chemical and pharmacological properties of a large number of oxotremorine analogues have been extensively studied (4, 5).

Compound BM-5 (Fig. 1A) is a member of a series consisting of 31 carboxamides recently synthesized by Prof. R. Dahlbom and his colleagues (6) and examined with respect to their tremorogenic/tremorolytic activity and for their effect on the contractions of isolated strips of guinea pig ileum (6).

BM-5 was a gift of Prof. Richard Dahlbom (Uppsala Sweden). Atropine sulfate and eserine sulfate were obtained from Sigma Chemical Company, (St. Louis, Mo.); carbamylcholine chloride was obtained from Siegfried AG (Zofingen, Switzerland), and hemicholinium-3 and oxotremorine sesquifumarat from (Aldrich, (Beerse, Belgium). All other chemicals were of reagent grade.

Competition between Compound BM-5 (10 nM–10 mM) and [^{3}H]3-QNB (0.2 nM) was studied using membranes

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[3] The abbreviations used are: ACh, acetylcholine; BM-5, *N*-methyl-*N*-(1-methyl-4-pyrrolidino-2-butynyl)acetamide; 3-QNB, 3-quinuclidinyl benzilate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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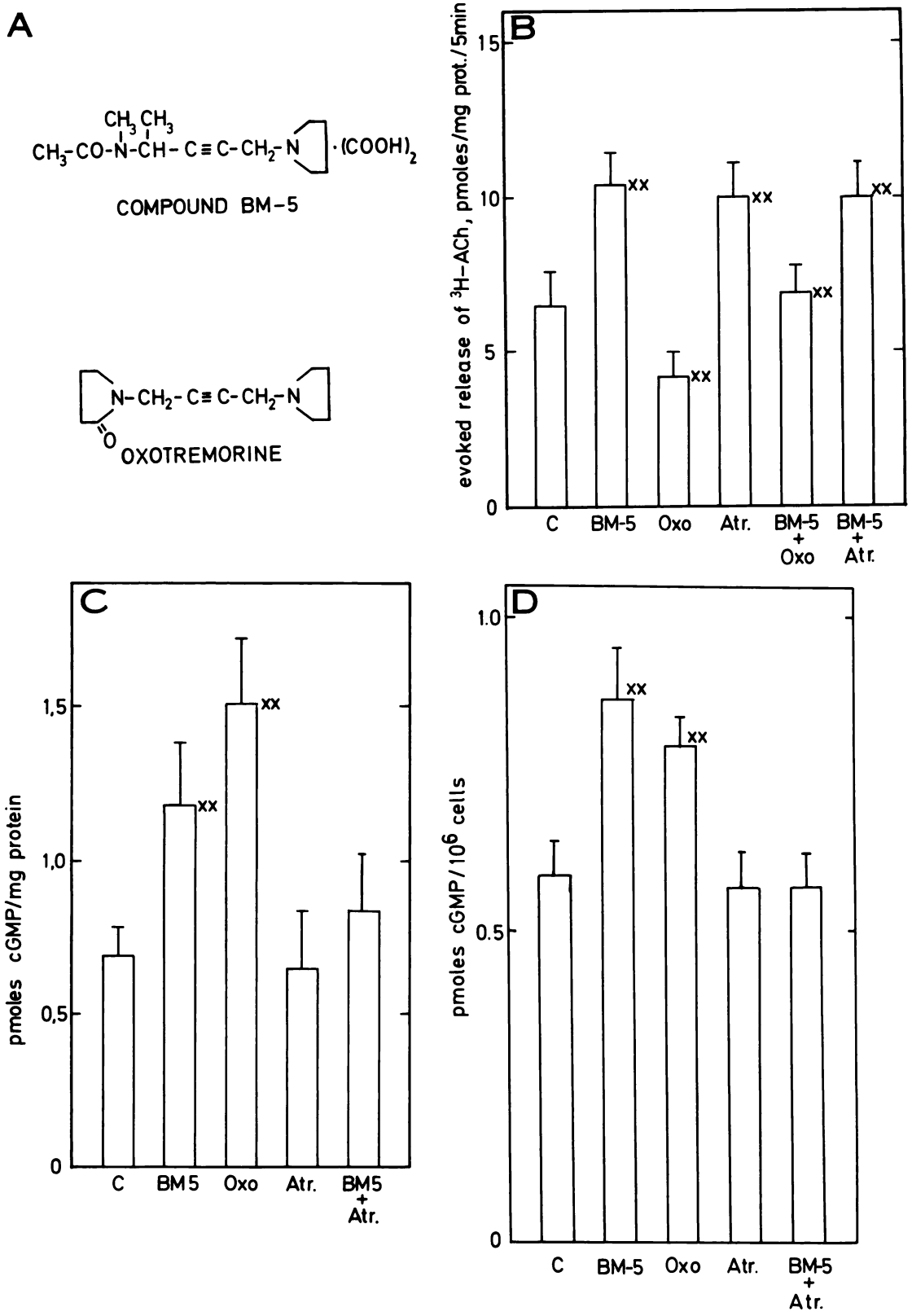

FIG. 1. *Structure of BM-5 and effects on rat hippocampal slices and human lymphocytes*

A. The structural formula of Compound BM-5. For comparison the structural formula of oxotremorine is also shown. Compound BM-5 was synthesized by Resul *et al.* (6).

B. The effect of BM-5, oxotremorine, and atropine on the potassium (25 mM)-evoked release of [^{3}H]ACh from slices of rat hippocampus. Control release (of [^{3}H]ACh evoked by high potassium, 25 mM) with no drug present (*C*) and in the presence of BM-5 (0.1 mM), oxotremorine (10 μM) (*oxo*), and atropine (1.0 μM) (*atr*). Values are means ± standard error of the mean for 6–10 samples assayed in duplicate. ××, Significantly different from control ($p < 0.05$).

prepared from rat hippocampus. Membranes were prepared from hippocampus of male Sprague-Dawley rats (200–250 g) by homogenization in Hepes (5 mM, pH 7.4)-buffered sucrose (10%, w/v) followed by centrifugation at 1,000 × *g* for 15 min. The resulting supernatant was centrifuged at 10,000 × *g* for 30 min, and the crude mitochondrial pellet obtained was resuspended in Krebs-Ringer's buffer [138 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $NaHPO_4$, 1 mM $CaCl_2$, 10 mM glucose, and 5 mM Hepes (pH 7.4)]. Incubations with [^{3}H]3-QNB (0.2 nM) and various concentrations of Compound BM-5 were carried out at 37° for 60 min and were terminated by filtration (2 sec) over Whatman GF/C glass-fiber filters. The filters were rinsed twice with 10 ml of cold buffer, dried, and counted with Lumagel scintillation cocktail in a Beckman scintillation spectrometer. Nonspecific binding of [^{3}H]3-QNB was defined as binding in the presence of atropine (10 μM). The binding data were analyzed by means of a nonlinear regression analysis, fitting it to a single binding isotherm as described earlier (7).

Rat hippocampi were cut into 0.4-mm thick slices with a McIlwain tissue chopper in a sagittal direction. The slices were incubated with choline (1 μM) and with [^{3}H] choline (60–90 Ci/mmole, New England Nuclear Corporation, Boston, Mass.) (50 nM) as a tracer in Krebs-Ringer's buffer for 60 min at 37° under continuous bubbling with 5% CO_2 in O_2 as described previously (8).

After labeling, the slices were washed and placed in Krebs-Ringer's buffer supplemented with eserine (10 μM). Under these conditions the spontaneous efflux of [^{3}H]ACh and [^{3}H]choline was studied for 20 min at 37°. Release of [^{3}H]ACh was evoked by high potassium concentration (25 mM). Isotonicity was held constant by equimolar substitution of potassium with sodium. The evoked release of [^{3}H]ACh and [^{3}H]choline was followed for 5 min in the presence and absence of the compounds studied.

The separation of [^{3}H]ACh and [^{3}H]choline was carried out by a modification of the method of Goldberg and McCaman (9), as described previously (10). In some experiments cyclic GMP was extracted from the slices according to the method of Folbergrová (11) and assayed with an acetylated radioimmunoassay according to the method of Brooker *et al.* (12).

Lymphocytes were prepared from leukocyte-rich plasma (13). For cyclic GMP measurements, 12 × 10^6 lymphocytes were incubated in 1-ml aliquots of Hanks' balanced salt solution supplemented with 3-isobutyl-1-methylxanthine (0.5 mM) for 15 min at 37°. The compounds studied were added, and incubation was continued for 1 min. The incubations were terminated by the addition of EDTA (20 mM) to give a final concentration of 4 mM and by placing the reaction tubes in a boiling water bath for 3 min. The boiled cell suspensions were sonicated and centrifuged for 10 min at 2600 × *g*. The supernatants were removed and the aliquots were analyzed with an acetylated cyclic GMP radioimmunoassay (12).

Male guinea pigs were stunned and bled, and the longitudinal muscle of the ileum with the myenteric plexus attached was dissected out at room temperature. A strip of the longitudinal muscle, about 3 cm in length, weighing 20–30 mg, was prepared from the ileum 10 cm proximal to the ilocecal sphincter. The strip was mounted in a 2-ml organ bath as previously described (10). The medium was Tyrode's solution of the following composition (millimolar): NaCl, 136.9; KCl, 2.7; $CaCl_2$, 1.8; $MgCl_2$, 0.5; $NaHCO_3$, 11.9; NaH_2PO_4, 0.4; and *d*-glucose, 5.6. The solution was gassed with 6.5% CO_2 in O_2.

The longitudinal muscle strip was mounted under a passive load of 5 mN and superfused at a rate of 1 ml/min with Tyrode's solution supplemented with hexamethonium (0.1 mM). For each of the four dose-response curves, BM-5 was added twice for 1 min at 6-min intervals. The contractile response is expressed relative to the maximal contraction elicited by carbamylcholine (1 μM) of each longitudinal muscle strip.

The guinea pig ileum longitudinal muscle preparation with the myenteric plexus attached was preincubated with choline (1 μM) and [^{3}H]choline (0.5 μM, 60–90 Ci/mmole; New England Nuclear Corporation) and mounted in an organ bath as previously described (10). During the preincubation the strips were field-stimulated at 0.2 Hz, and the Tyrode's medium contained eserine (10 μM). After the preincubation, hemicholinium (10 μM) was added to the medium. The strips were field-stimulated via two platinum ring electrodes, 4 cm apart at the top and bottom of the preparation, with 150 shocks at 1 Hz, 0.5 msec in duration and with 100 V and 140 mamp between the electrodes. The [^{3}H]ACh collected in the superfusion fractions was separated from [^{3}H]choline and other [^{3}H]choline derivatives by the method of Goldberg and McCaman (9) as previously described (10). The secretion of [^{3}H]ACh in the presence of BM-5 is expressed relative to the secretion of [^{3}H]ACh in control experiments, which were carried out with the same experimental protocol (14).

BM-5 (cf. formula, Fig. 1A) binds to muscarinic receptors labeled by [^{3}H]3-QNB in the rat hippocampus. The equilibrium binding constant determined at 37° was 3.07 ± 0.09 μM ($n = 30$) (Table 1). At the concentration used (0.2 nM), [^{3}H]3-QNB is known to label only postsynaptic receptors (cf. ref. 2). Compound BM-5 in concentrations above 10^{-3} M fully displaced the specifically bound [^{3}H] 3-QNB.

Compound BM-5 behaves like a presynaptic antagonist when, like atropine, it enhances potassium depolarization-evoked release of [^{3}H]ACh from slices of rat hippocampus (Fig. 1B). This effect is exerted at muscarinic receptors, since it is antagonized by oxotremorine (Fig.

C. The effect of BM-5 (0.1 mM), oxotremorine (0.1 mM), and atropine (1 μM) on cyclic GMP levels in slices of rat hippocampus. Slices of rat hippocampus were incubated in Krebs-Ringer's buffer with 3-isobutyl-1-methylxanthine (0.5 mM) for 15 min at 37°. The compounds studies were added, and incubation was continued for 2 min. Values are means standard error of the mean for three samples assayed in triplicate. ××, Significantly different from control ($p < 0.05$).

D. The effect of BM-5, oxotremorine, and atropine on cyclic GMP levels in human lymphocytes. Values are means ± standard error of the mean for three samples assayed in triplicate. ××, Significantly different from the control ($p < 0.05$).

TABLE 1

Data on the potency of Compound BM-5 at pre- and postsynaptic muscarinic receptors

Tissue	Type of measurement	Assumed site of action	EC_{50}	IC_{50}
			μM	μM
Rat hippocampus	Competition for [^{3}H]3-QNB binding	Postsynaptic	—	3.1 ± 0.1
Rat hippocampus	Enhancement of [^{3}H]-ACh release	Presynaptic	7.1 ± 0.5	
Guinea pig ileum	Contraction of longitudinal muscle	Postsynaptic	0.5 ± 0.2	
Guinea pig ileum	Depression of [^{3}H]ACh release in the absence of eserine	Presynaptic	0.5 ± 0.1	
Guinea pig ileum	Enhancement of [^{3}H]ACh release in the presence of eserine	Presynaptic	3.2 ± 0.4	

1B). The enhancement of evoked [^{3}H]ACh release is half-maximal at a 7.1 ± 0.5 μM concentration of BM-5 (Table 1).

Stimulation of cyclic GMP synthesis shows a good correlation with the muscarinic postsynaptic agonist properties of cholinergic ligands at synapses in sympathetic ganglia, heart, and cerebral cortex and in non-nerve tissues (cf., for example, ref. 15). Behaving like a muscarinic agonist, BM-5 induced an increase in the cyclic GMP levels in hippocampal slices (Fig. 1C). However, in a slice preparation, it is always difficult to judge whether the effects of a compound are exerted directly or indirectly through the release of other compounds. We therefore chose to study the "postsynaptic effects" of BM-5 in a non-nerve tissue using human lymphocytes, which are known to respond to binding of muscarinic agonists by elevated cyclic GMP levels (16).

Figure 1D shows that BM-5, acting at muscarinic receptors, stimulated cyclic GMP synthesis in human lymphocytes, thus behaving in this system, which lacks presynaptic receptors, as a postsynaptic muscarinic agonist.

In the guinea pig ileum longitudinal muscle-myenteric plexus preparation, BM-5 caused contraction of the prep-

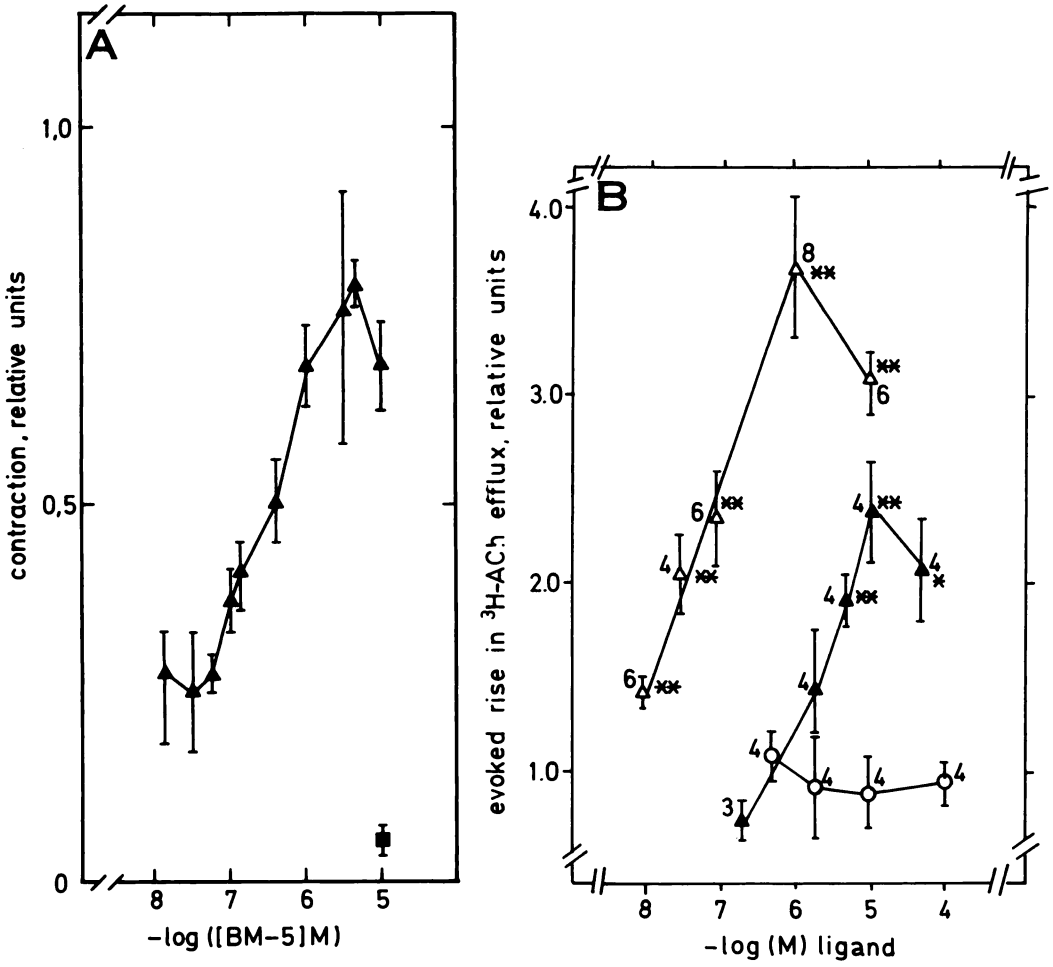

FIG. 2. *Effect of BM-5 on guinea pig ileum longitudinal muscle preparations*

A. Contraction of guinea pig ileum longitudinal muscle myenteric plexus preparation by BM-5 (▲) and BM-5 plus atropine (1 μM) (■).

B. The effect of BM-5 on the electrically evoked release of [^{3}H]ACh from guinea pig ileum longitudinal muscle myenteric plexus preparation in the presence of eserine (10 μM). △, Atropine; ▲, BM-5; ○, oxotremorine. Values are means ± standard error of the mean of four observations. ** and *, Significantly different from control (release of [^{3}H]ACh evoked by stimulation with no drug present) ($p < 0.01$ and $p < 0.05$, respectively).

aration in a dose-dependent manner (EC_{50} = 0.49 ± 0.18 μM; n = 4) (Table 1; Fig. 2A). This effect, which was studied in the presence of hexamethonium (0.1 mM), was completely blocked by atropine (1 μM) (Fig. 2A). When the presynaptic activity of this compound was examined in the absence of eserine, it depressed the electrically evoked release of [^{3}H]ACh with an EC_{50} of 0.54 ± 0.10 μM (n = 2) (stimulation parameters were 0.5 Hz, 0.5-msec duration, 150 shocks). However, when the presynaptic activity of BM-5 was examined in the presence of eserine (10 μM), it enhanced the electrically evoked (stimulation 1 Hz, 0.5-msec duration, 150 shocks) release of [^{3}H]ACh in a dose-dependent manner (EC_{50} = 3.2 ± 0.4 μM; n = 6) (Fig. 2B). Thus, under conditions of partial inhibition of the ACh esterase BM-5 acted as a presynaptic antagonist in this system and enhanced evoked release of [^{3}H]ACh (Fig. 2B).

Compound BM-5, an oxotremorine analogue, which recently has been synthesized and characterized by Resul *et al.* (6), appears to fulfill several pharmacological and biochemical requirements for a muscarinic ligand *in vitro*. It competes for binding of the specific muscarinic antagonist [^{3}H]3-QNB to the same extent as does the classical muscarinic antagonist atropine (2). It contracts the guinea pig ileum (6) (Fig. 2A) in a dose-dependent manner, and this action can be blocked by atropine.

Stimulation of cyclic GMP synthesis by muscarinic cholinergic ligands in nerve and non-nerve tissues correlates well with the postsynaptic, agonist-like potency of these compounds (cf. refs. 15 and 16). In slices of the rat hippocampus as well as in human lymphocytes (Fig. 1D), Compound BM-5, like oxotremorine, stimulated cyclic GMP synthesis. These effects could also be blocked by atropine.

The lack of effects of BM-5 on the resting release of [^{3}H]ACh, as well as the fact that it alone contracted the preparation in the presence of hexamethyonium, indicates that its postsynaptic agonist effect cannot be explained by assuming that it releases ACh from the nerves; rather it appears to reflect a direct stimulation of postsynaptic muscarinic receptors.

The presynaptic actions of Compound BM-5 are similar to those of the classical muscarinic antagonist, atropine. In the presence of eserine (10 μM), BM-5 enhanced the K^+ depolarization-evoked release of [^{3}H]ACh from the rat hippocampus (Fig. 1B) and the electrical stimulation-evoked release of [^{3}H]ACh from the guinea pig ileum-myenteric plexus (Fig. 2B) preparation.

The presynaptic "antagonist" postsynaptic agonist-like properties of Compound BM-5 reflect the fact that it is an unusual muscarinic ligand which, in inducing the contractile response, behaves as a partial agonist (6). *In vivo*, BM-5 is a tremorolytic agent rather than a tremorogenic agent, which suggests that in brain regions which govern the tremor response it may behave mostly as an antagonist.

Although the potency of BM-5 at muscarinic receptors is several orders of magnitude lower than that of the commonly used antagonists atropine (cf. ref. 17) and scopolamine, and its potency is about 10-fold lower than that of oxotremorine (cf. ref. 14), we believe that this compound is of general interest, because it behaves at the same synapse (in the hippocampus and in the myenteric plexus, in the presence of eserine, i.e., when levels of endogenous agonist are elevated), as a presynaptic antagonist and as a postsynaptic agonist (cf. Table 1). Thus it affords an unattenuated enhancement of the signal across the muscarinic synapse. Compounds which enhance cholinergic transmission would be of therapeutic value in the treatment of several diseases, e.g., Huntington's chorea (18) and Alzheimer-type dementia (19), where lowered cholinergic function has been reported from autopsy studies, and drug therapies based on increased availability of precursor (choline) are suggested because appropriate cholinergic agonists are lacking.

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