Evaluation of the cholinomimetic actions of trimethylsulfonium, a compound present in the midgut gland of the sea hare *Aplysia brasiliana* (Gastropoda, Opisthobranchia)

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Abstract

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Trimethylsulfonium, a compound present in the midgut gland of the sea hare *Aplysia brasiliana*, negatively modulates vagal response, indicating a probable ability to inhibit cholinergic responses. In the present study, the pharmacological profile of trimethylsulfonium was characterized on muscarinic and nicotinic acetylcholine receptors. In rat jejunum the contractile response induced by trimethylsulfonium ($pD_2 = 2.46 \pm 0.12$ and maximal response $= 2.14 \pm 0.32$ g) was not antagonized competitively by atropine. The maximal response (E_{max}) to trimethylsulfonium was diminished in the presence of increasing doses of atropine ($P<0.05$), suggesting that trimethylsulfonium-induced contraction was not related to muscarinic stimulation, but might be caused by acetylcholine release due to presynaptic stimulation. Trimethylsulfonium displaced [^{3}H]-quinuclidinyl benzilate from rat cortex membranes with a low affinity ($K_i = 0.5$ mM). Furthermore, it caused contraction of frog rectus abdominis muscles ($pD_2 = 2.70 \pm 0.06$ and $E_{max} = 4.16 \pm 0.9$ g), which was competitively antagonized by d-tubocurarine (1, 3 or 10 µM) with a pA_2 of 5.79, suggesting a positive interaction with nicotinic receptors. In fact, trimethylsulfonium displaced [^{3}H]-nicotine from rat diaphragm muscle membranes with a K_i of 27.1 µM. These results suggest that trimethylsulfonium acts as an agonist on nicotinic receptors, and thus contracts frog skeletal rectus abdominis muscle and rat jejunum smooth muscle via stimulation of postjunctional and neuronal prejunctional nicotinic cholinoreceptors, respectively.

Introduction

The opisthobranch gastropod molluscs, belonging to the family Aplysiidae, commonly called sea hares, are known to possess cholinomimetic principles in the midgut gland (1,2). Many of these compounds, like murexine (3), dihydromurexine, senecioylcholine and tigloylcholine (4), act as agonists or antagonists on nicotinic acetylcholine receptors. Extracts from the midgut gland of the sea hare *Aplysia brasiliana*, which contain tetramethylammonium and trimethylsulfonium chloride (5), contract invertebrate muscles and prevent vagus-induced toad heart arrest (6). Preliminary studies suggest that trimethylsulfonium is the active principle of the extract (7).

The aim of the present study was to extend the studies with trimethylsulfonium looking for its cholinergic properties. Thus, we studied its effect on the binding to muscarinic and nicotinic receptors, as well as on classical cholinergic bioassay models. Binding studies were performed on rat cerebral cortex and diaphragm muscle membranes, while bioassays were done on rat jejunum and frog rectus abdominis muscle.

Material and Methods

Drugs and chemicals

Trimethylsulfonium (C_3H_9SCl, 112.4 g) has been isolated from the midgut gland of *A. brasiliana* in its chloride form; however, it has also been synthesized in the iodide form (C_3H_9SI, 203.9 g; Aldrich Chemical Company, Inc., Milwaukee, WI, USA), which was used in the present study. The following drugs were also used: carbachol, d-tubocurarine, acetyl-ß-methylcholine (methacholine), (-)-nicotine, atropine, phenylmethylsulfonylfluoride (PMSF), EDTA and Trizma Base (Sigma, St. Louis, MO, USA), (-)-N-methyl-[3H]-nicotine (81.5 Ci/mmol; NEN® Sciences Products, Inc., Boston, MA, USA),

[3H]-quinuclidinyl benzilate ([3H]-QNB, 42 Ci/mmol; ICN Biomedicals, Inc., Costa Mesa, CA, USA), and the salts (Quimitra S/A, Rio de Janeiro, RJ, Brazil).

Functional studies

Effect on muscarinic acetylcholine receptors. Male Wistar rats (*Rattus norvegicus*, 4-5 months old) were killed by decapitation. Tissues were prepared as described by Kobashi et al. (8). A segment of approximately 5 cm of the jejunum was isolated and washed to remove its contents with Tyrode solution (137 mM NaCl, 2.7 mM KCl, 0.5 mM NaH_2PO_4, 2.2 mM $MgCl_2$, 3.6 mM $CaCl_2$, 11.9 mM $NaHCO_3$ and 5.5 mM glucose). Jejunum segments (2 cm in length), resuspended in Tyrode solution (35°C), were equilibrated for 30 min prior to the beginning of the experimental protocol. Isometric muscular contractions were recorded on a Narco polygraph (Narco BioSystems, Houston, TX, USA) through a Narco force transducer (model F-60) under an initial tension of 1 g.

The antagonistic effect of trimethylsulfonium (1, 3 and 10 mM) on muscarinic receptors was evaluated against concentration-response curves to acetyl-ß-methylcholine (methacholine, 0.1 µM to 0.3 mM), and compared to atropine (0.1, 1 and 10 nM). The agonistic effect of trimethylsulfonium on muscarinic receptors was tested through cumulative concentration-response curves (0.05 to 12.8 mM) in the absence or in the presence of atropine (1, 10 or 100 nM). In these experiments, we obtained three consecutive and cumulative curves in response to the agonists at 1-h intervals. The last curve was obtained in the presence of a single concentration of the antagonist added to the bath immediately after completing the second curve, i.e., antagonists were incubated for 1 h before agonist testing. After each curve, the tissues were washed with Tyrode solution.

Effect on nicotinic acetylcholine receptors. Frogs (*Rana catesbeiana*) were killed by decerebration. Each rectus abdominis muscle was removed, cut longitudinally into four strips, and transferred to a 7-ml organ bath containing Ringer solution (109.1 mM NaCl, 4.0 mM KCl, 4.2 mM NaHCO$_3$, 1.6 mM CaCl$_2$ and 3.9 mM glucose) at room temperature (25°C), under a tension of 1.5 g. The isometric muscular responses were recorded on a Narco polygraph through a Narco force transducer (model F-60).

The agonistic effect of trimethylsulfonium (0.3 to 76.8 mM) on nicotinic receptors was evaluated through concentration-response curves, comparing its efficacy to the full-agonist carbachol (8 to 512 μM). In these experiments we obtained three consecutive and cumulative curves for the agonists at 1-h intervals. The last curve was obtained in the presence of a single concentration of the antagonist d-tubocurarine (1, 3 and 10 μM), added to the bath immediately after completing the second curve. After each curve, the tissues were washed with Tyrode solution.

Acetylcholine receptor binding assays. Male Wistar rats (4-5 months old) were killed by decapitation. Membranes rich in muscarinic receptors were obtained from rat brain cortex. The cortex was homogenized (Polytron Brinkmann homogenizer) in 20 ml of ice-cold sucrose solution (0.32 M), centrifuged (500 *g*, 10 min, 4°C) and the pellet was discarded. The supernatant was further centrifuged (40,000 *g*, 10 min, 4°C), and the resulting pellet was resuspended in 5 volumes of the assay buffer (50 mM Na$^+$/K$^+$ phosphate, pH 7.4). Aliquots of the crude membrane preparation were stored at -70°C for up to one week.

Membranes rich in nicotinic receptors were obtained from rat diaphragm muscle. The muscle was isolated and homogenized in ice-cold 0.4 M Tris-HCl buffer, pH 7.5, containing 10 mM EDTA and 0.1 mM PMSF and centrifuged (5,000 *g*, 10 min, 4°C), and

the pellet was discarded. The supernatant was further centrifuged (40,000 *g*, 10 min, 4°C) and the resulting pellet was resuspended in 10 volumes of assay buffer (20 mM Tris-HCl, pH 7.5, containing 0.1 mM EDTA). Aliquots of the crude membrane preparation were stored at -70°C for up to 24 h.

Protein concentration was estimated by the method of Bradford (9) using bovine serum albumin as standard.

Competition binding assays were performed with a radioligand (1 nM [^{3}H]-QNB, and 8 nM [^{3}H]-nicotine) for muscarinic and nicotinic receptors, respectively. For nicotinic binding assays, bovine serum albumin (0.1 mg/ml) was added to the Tris-HCl assay buffer. Nonspecific binding was defined as that occurring in the presence of atropine (2 mM) or nicotine (10 mM) for muscarinic and nicotinic receptors, respectively.

After 1-h incubation at room temperature, the samples were immediately filtered through Whatmann GF/B glass-fiber filters. The filters were washed three times with 4 ml of ice-cold buffer and placed in vials containing 5 ml of scintillation cocktail (Ecolume, ICN Biomedicals, Inc.) and radioactivity was measured with a scintillation counter (Tri-Carb 2100TR, Packard, Camberra Co., Meriden, CT, USA).

Statistical analysis

The results are reported as means ± SEM for both the competition and the concentration-response curves.

The IC$_{50}$ was obtained by nonlinear regression with a 95% confidence interval (95% CI). For the binding assays, the IC$_{50}$ of trimethylsulfonium and atropine were compared by the Student *t*-test (P<0.05). The dissociation constant (K_i) was obtained by the following formula:

$$K_i = \frac{IC_{50}}{1 + [L]K_D^{-1}}$$

where [L] is the concentration of the radio-

ligand used and K_D its dissociation constant. The K_D used for [³H]-QNB was 0.06 nM, according to Yamamura and Snyder (10), and the K_D used for [³H]-nicotine was 1 μM, according to Brioni et al. (11). The selectivity of trimethylsulfonium was calculated by the ratio of the inhibition constants for muscarinic and nicotinic receptors.

For the functional studies, E_{max} and pD_2 values are expressed as means ± SEM calculated from individual experiments. The E_{max} obtained for methacholine, carbachol and trimethylsulfonium in rat jejunum or frog rectus abdominis were compared by the Student *t*-test (P<0.05). The E_{max} obtained for control and treated preparations were compared by one-way analysis of variance followed by the Newman-Keuls test.

The antagonist affinities (atropine and d-tubocurarine) were determined by Equation I proposed by Arunlakshana and Schild (12). According to these investigators, if the blockade is competitive, a plot of the logarithm of (dose ratio-1) against the negative logarithm of the molar concentration of the blocker (B) will yield a straight line with a slope of one and intercept along the abscissa at pA_2, which under equilibrium conditions is equal to the negative logarithm of the dissociation constant of the antagonist (Equation II).

$$\log (DR-1) = \eta \log [B] + \log K_B \quad (I)$$
$$pA_2 = -\log K_B \quad (II)$$

Schild analysis was performed by linear regression, and the slope of the straight line was obtained with SEM. The Graphpad Prism program was used for curve construction and statistical analysis (Intuitive Software for Science, San Diego, CA, USA).

Results

Effect of trimethylsulfonium on muscarinic receptors

In order to determine if trimethylsulfonium acts on muscarinic receptors, as suggested by the inhibition of vagus-induced toad heart arrest (6), its ability to displace [³H]-QNB was tested (Figure 1). Trimethylsulfonium (K_i = 0.5 mM) displaced [³H]-QNB with a very low affinity compared to atropine (0.6 nM).

The functional agonistic and antagonistic properties of trimethylsulfonium on muscarinic receptors were assessed in the rat isolated jejunum. Cumulative additions of the compound caused concentration-dependent contractions, with a pD_2 and E_{max} of 2.46 ± 0.12 and 2.14 ± 0.32 g (N = 9), respectively (Figure 2). The curve for trimethylsulfonium was concentration-dependently reduced by atropine (1, 10 and 100 nM) without changes in pD_2 values. In contrast, atropine antagonized methacholine-induced contractions in a purely competitive manner, yielding a Schild plot regression line with a pA_2 value of 9.58 and a slope of 0.88 ± 0.09 (correlation coefficient 0.95, N = 12). Furthermore,

Figure 1. Inhibition of specific [³H]-quinuclidinyl benzilate (QNB) binding to membranes isolated from rat cortex by atropine (filled squares, N = 8) or trimethylsulfonium (open squares, N = 8). Membranes were incubated with [³H]-QNB and unlabeled compounds for 1 h at room temperature in Na⁺/K⁺ phosphate buffer. Specific binding was calculated by subtracting the nonspecific binding measured in the presence of 2 mM atropine from total binding (T). Data are reported as means ± SEM.
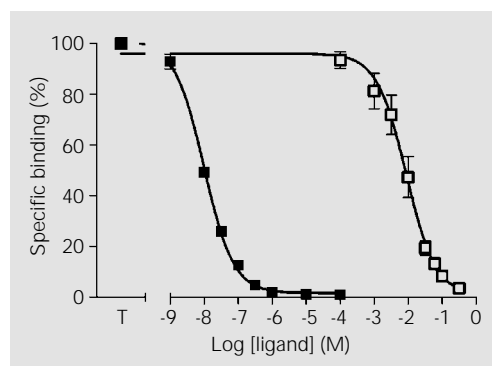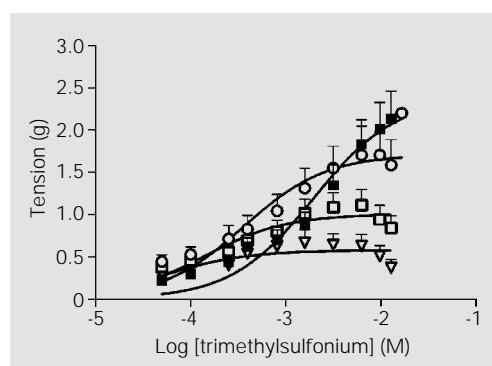

Figure 2. Cumulative concentration-response curves (1-h interval) for trimethylsulfonium in rat jejunum, in the absence (filled squares, N = 9) and in the presence of 1 nM atropine (circles, N = 7), 10 nM atropine (open squares, N = 8) and 100 nM atropine (inverted triangles, N = 5). The antagonist was applied 1 h before the agonist. Data are reported as means ± SEM.

at 1, 3 or 10 mM, trimethylsulfonium failed to affect rat jejunum responsiveness to the selective muscarinic receptor agonist methacholine. The pD_2 and E_{max} values obtained for methacholine in the absence or presence of 10 mM trimethylsulfonium were 6.06 ± 0.11 and 3.12 ± 0.18 g (N = 12) and 5.93 ± 0.31 and 2.43 ± 0.25 g (N = 4), respectively.

Effect of trimethylsulfonium on nicotinic receptors

Trimethylsulfonium and nicotine displaced [³H]-nicotine binding from rat diaphragm muscle membranes and the K_i values for both ligands were of the same order of magnitude, i.e., 27.1 and 6.6 µM, respectively (Figure 3), suggesting that trimethylsulfonium has a direct effect on nicotinic receptors.

In fact, trimethylsulfonium contracted frog skeletal muscle in a concentration-dependent manner, with a pD_2 value of 2.70 ± 0.06 and an E_{max} similar to carbachol (4.16 ± 0.90, N = 12, and 3.32 ± 0.73 g, N = 12, respectively) (Figure 4A). The nicotinic blocker d-tubocurarine antagonized trimethylsulfonium and carbachol concentration-response curves in a competitive fashion, yielding a Schild plot regression line with pA_2 values of 5.79 and 5.81, respectively, and a slope of 1.12 ± 0.17 (correlation coefficient 0.81, N = 23) and 0.98 ± 0.15 (correlation coefficient 0.81, N = 20), respectively (Figure 4B).

Discussion

In the present study we examined the action of trimethylsulfonium on cholinergic systems in order to assess its functional and binding properties in terms of muscarinic and nicotinic receptors.

Since trimethylsulfonium antagonizes heart vagus nerve effect (6,7), the first hypothesis was that trimethylsulfonium blocks muscarinic receptors. However, since tri-

methylsulfonium displaced [³H]-QNB binding with a very low affinity and it did not block the effect of methacholine on rat jejunum, this initial hypothesis was not confirmed. On the other hand, trimethylsulfonium itself promoted a concentration-dependent contraction of the rat jejunum, which was not competitively inhibited by atropine, indicating that trimethylsulfonium acts by a mechanism independent of muscarinic receptors.

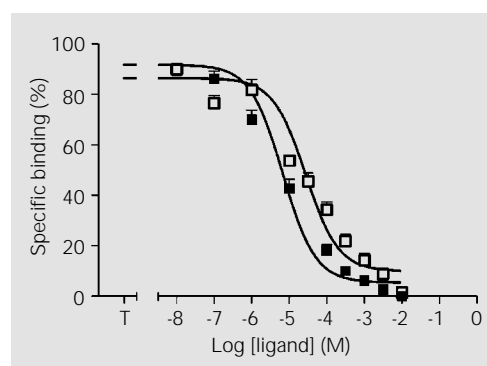It is well known that contraction of intestinal smooth muscle can be elicited by sev-

Figure 3. Inhibition of specific [³H]-nicotine binding to membranes isolated from rat diaphragm by unlabeled nicotine (filled squares, N = 8) or trimethylsulfonium (open squares, N = 8). Membranes were incubated with [³H]-nicotine and unlabeled compounds for 1 h at room temperature in Tris-HCl buffer. Specific binding was calculated by subtracting the nonspecific binding measured in the presence of unlabeled 10 mM nicotine from total binding (T). Data are reported as means ± SEM.
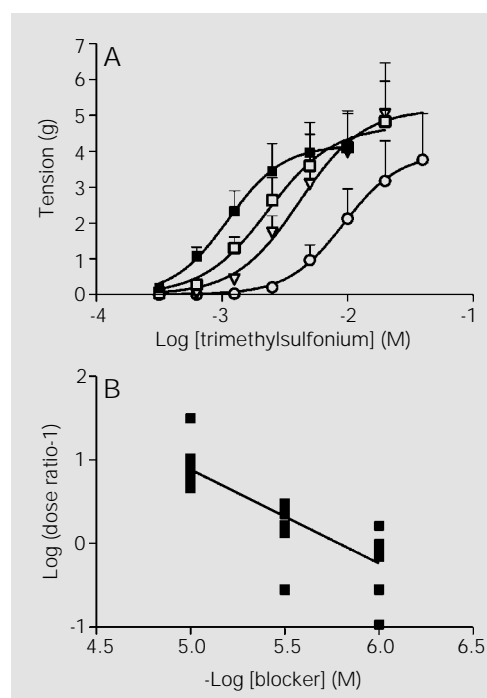

Figure 4. A, Cumulative concentration-response curves (1-h interval) for trimethylsulfonium in frog rectus abdominis muscle, in the absence (filled squares, N = 12) and in the presence of 1 µM d-tubocurarine (open squares, N = 9), 3 µM d-tubocurarine (inverted triangles, N = 8) and 10 µM d-tubocurarine (circles, N = 7). The antagonist was applied 1 h before the agonist. Data are reported as means ± SEM. B, Schild plot of the antagonism of the response of trimethylsulfonium by d-tubocurarine. The line was calculated by linear regression analysis.

eral neurotransmitters acting directly on the muscle or through the enteric nervous system. Acetylcholine, acting on nicotinic acetylcholine receptors, is the main excitatory neurotransmitter in the myenteric plexus (13). Therefore, trimethylsulfonium could act presynaptically by releasing acetylcholine, which will act on muscarinic receptors of the muscle. In this case, atropine would not be able to displace trimethylsulfonium from its receptor, but would block the effect of endogenous acetylcholine acting on muscarinic receptors. A similar effect has been observed in the prostatic portion of the rat vas deferens where stimulation of nicotinic presynaptic cholinoreceptors induced the release of neurotransmitters, and the blockade of postsynaptic receptors only reduced the maximal response to nicotinic agonists (14). However, since the release of acetylcholine was not directly measured it is not possible to rule out that trimethylsulfonium simultaneously releases other neurotransmitters which contract the rat jejunum preparation, or even acts on smooth muscle cells through a mechanism independent of muscarinic stimulation. Since atropine only reduced the maximal response to trimethylsulfonium in rat jejunum, we decided to test if this drug was able to bind to and stimulate nicotinic receptors.

In fact, trimethylsulfonium displaced [^{3}H]-nicotine binding in a manner similar to nicotine. In addition, the trimethylsulfonium-induced concentration-response curve for rectus abdominis muscle was antagonized by d-tubocurarine in a competitive manner. The pA$_2$ value for d-tubocurarine determined using trimethylsulfonium as agonist did not differ from that obtained with carbachol (present study), and was only slightly lower than the values estimated by other authors using frog cutaneous pectoris muscle (pA$_2$ = 6.4) (15,16). It is interesting to note that the presence of the three methyl radicals in the trimethylsulfonium molecule favors nicotinic over muscarinic action. N-methyl-carbamylcholine, a carbachol analogue, is selective for nicotinic receptors, while carbachol binds to both nicotinic and muscarinic receptors (17).

Acknowledgments

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