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Identification of rat brain muscarinic M_4 receptors coupled to cyclic AMP using the selective antagonist muscarinic toxin 3

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Abstract

In membranes of olfactory tubercle and striatum, the selective muscarinic M_4 receptor antagonist muscarinic toxin 3 completely antagonized the acetylcholine-induced inhibition of forskolin- and dopamine D_1 receptor-stimulated cyclic AMP formation with K_i values of 7 and 4 nM, respectively. In olfactory bulb, where acetylcholine stimulated basal adenylyl cyclase activity and inhibited forskolin-stimulated enzyme activity, muscarinic toxin 3 caused a partial antagonism of both acetylcholine effects with high potencies (K_i values = 4–6 nM). In frontal cortex, muscarinic toxin 3 counteracted the acetylcholine-induced potentiation of corticotropin-releasing hormone-stimulated cyclic AMP with a K_i of 58 nM, which is close to the toxin affinity for the muscarinic M_1 receptor. In the same brain region, the acetylcholine inhibition of forskolin-stimulated enzyme activity was not affected by muscarinic toxin 3. In microdissected regions of the hippocampus, a significant portion (33–48%) of the acetylcholine inhibition of forskolin-stimulated adenylyl cyclase activity was blocked by muscarinic toxin 3 with K_i values (6–8 nM) consistent with the involvement of muscarinic M_4 receptors. These data show that muscarinic toxin 3 discriminates between adenylyl cyclase-coupled muscarinic receptors and demonstrate the utility of the toxin in identifying the relative contribution by the muscarinic M_4 receptor subtype.

Keywords: Muscarinic toxin 3; Muscarinic receptor subtype; Adenylyl cyclase; Brain, rat

1. Introduction

The regulation of adenylyl cyclase activity constitutes one of the major signalling pathways of acetylcholine muscarinic receptors (Nathanson, 1987; Caufield, 1993). Among the five receptor subtypes so far cloned, the muscarinic M_2 and M_4 receptors have been shown to be preferentially coupled to cyclic AMP regulation through the interaction with G proteins of the Gi/Go family (Peralta et al., 1988; Hulme et al., 1990). However, there is evidence that muscarinic M_1 and M_3 receptors can also affect cyclic AMP production, either by direct interaction with the stimulatory G protein G_s (Burford and Nahorski, 1996) or through the activation of Ca^{2+}-dependent pathways (Felder et al., 1989; Tachado et al., 1994; Esqueda et al., 1996). Thus, in tissues expressing a heterogeneous muscarinic receptor population, more than one receptor subtype may participate in the regulation of cyclic AMP signalling.

The brain contains all the five muscarinic receptor subtypes which are differently localized in neuronal circuits (Buckley et al., 1988; Levey et al., 1991). In light of the relevant role played by cyclic AMP in the modulation of synaptic transmission and neuronal activity, pharmacological studies have attempted to identify the muscarinic receptor subtype(s) regulating adenylyl cyclase activity in the different brain areas. It has been proposed that the cholinergic inhibition of adenylyl cyclase activity is mediated by muscarinic M_4 receptors in rat striatum (Ehlert et al., 1989; McKinney et al., 1989; Olianas and Onali, 1991) and cerebral cortex (McKinney et al., 1991) and by muscarinic M_2 receptors in the brainstem (McKinney et al., 1989). In addition, in the rat olfactory bulb cholinergic agonists have been found to stimulate adenylyl cyclase activity through the activation of a heterogeneous receptor population predominantly composed by the muscarinic M_4 subtype (Olianas and Onali, 1991), whereas in frontal

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cortex the muscarinic M_1 receptor subtype appears to mediate the cholinergic potentiation of adenylyl cyclase activity stimulated by corticotropin-releasing hormone (Onali and Olianas, 1996). However, in these studies the receptor characterization has been accomplished by using muscarinic receptor antagonists with limited subtype selectivity. For instance, none of the drug employed clearly discriminated between muscarinic M_2 and M_4 receptors. Therefore, the attribution of one response to either one of these subtypes could be disputed, especially when the receptors are coexpressed at a similar level.

The recent isolation of muscarinic toxin 3, a peptide toxin from green mamba venom with high selectivity for the cloned human muscarinic M_4 receptor (Karlsson et al., 1994; Jolkkonen et al., 1994), has offered the possibility of using a new pharmacological tool to identify the functional responses mediated by the native muscarinic M_4 receptor. Muscarinic toxin 3 was previously found to block the adenylyl cyclase-coupled muscarinic receptors of rat striatum with about 100-fold higher potency than the muscarinic M_2 receptors of rat myocardium, indicating that the toxin is an antagonist with a remarkable ability to discriminate between native muscarinic M_4 and M_2 receptor subtypes (Olianas et al., 1996).

In the present study, we employed muscarinic toxin 3 to assess the relative contribution of the muscarinic M_4 receptors in the regulation of adenylyl cyclase in various brain areas known to coexpress multiple muscarinic receptor subtypes.

2. Materials and methods

2.1. Materials

[α-^{32}P]ATP (30–40 Ci/mmol) and [2,8-^{3}H]cyclic AMP (25 Ci/mmol) were obtained from NEN-Du Pont (Bad Homburg, Germany). Forskolin and (±)-6-chloro-7,8-dihydroxy-3-allyl-1-phenyl-2,3,4,5-tetrahydro-1*H*-3-benzazepine hydrobromide ((±)-chloro-APB HBr) were purchased from Calbiochem (La Jolla, CA, USA) and Research Biochemicals International (Natick, MA, USA), respectively. Corticotropin-releasing hormone (CRH) was from Peninsula (Merseyside, UK). Muscarinic toxin 3 was purified from the venom of *Dendroaspis angusticeps* according to the procedure of Jolkkonen et al. (1994). Acetylcholine chloride, carbachol chloride, physostigmine hemisulphate and the other reagents used were from Sigma (St. Louis, MO, USA).

2.2. Tissue preparation

Male albino Sprague–Dawley rats (Charles River, Italy) weighing 150–250 g were used. The animals were killed by rapid decapitation with a guillotine and the brains were removed and placed on a cold plate. The brain regions were rapidly dissected according to published procedures (Robertson et al., 1989). Following dissection, the hippocampus was cut into transverse slices (300 μm thick) with a mechanical tissue slicer. By using a stereoscopic microscope equipped with a diascopic illuminator, each slice was dissected into three regions: dentate gyrus, CA1 and CA2/CA3. Olfactory bulbs and frontal cortex were homogenized by hand in 10 volumes (v/w) of ice-cold hypotonic buffer containing 10 mM HEPES/NaOH, 1 mM EGTA, and 1 mM $MgCl_2$ (pH 7.4). The homogenate was diluted and centrifuged at $27\,000 \times g$ for 20 min at 4°C. The pellet was resuspended in the same buffer and centrifuged as above. The final pellet was resuspended to a protein concentration of 1.0–1.5 mg/ml, incubated for 10 min in an ice-bath and used for the adenylyl cyclase assay. Striata and other brain regions were homogenized in 10 volumes of ice-cold homogenization buffer containing 0.32 M sucrose using a teflon/glass tissue grinder. The homogenates were diluted and centrifuged at $1000 \times g$ for 10 min at 4°C. The supernatant was aspirated and centrifuged at $11\,000 \times g$ for 20 min at 4°C. The pellet (P2 fraction) was resuspended by repeated aspiration through a plastic pipette in 30–50 volumes of buffer with no sucrose. For measurements of dopamine D_1 receptor-stimulated adenylyl cyclase activity, the P2 fraction of rat striatum was incubated for 20 min in ice-cold hypotonic buffer to favor synaptosomal lysis and was then used for the enzyme assay without any further treatment. The P2 fractions of other brain regions were resuspended in hypotonic buffer and centrifuged at $27\,000 \times g$ at 4°C and the final pellets were resuspended in the same buffer.

2.3. Adenylyl cyclase assay

The enzyme activity was assayed in a 100 μl reaction mixture containing 50 mM HEPES/NaOH (pH 7.4), 2.3 mM $MgCl_2$, 0.3 mM EGTA, 0.2 mM [α-^{32}P]ATP (45–60 cpm/pmol), 0.5 mM [^{3}H] cyclic AMP (150 cpm/nmol), 50 μM GTP, 1 mM 3-isobutyl-1-methylxanthine, 5 mM phosphocreatine, 50 U/ml of creatine kinase, 50 μg of bovine serum albumin, 10 μg of bacitracin, 10 kallikrein inhibitor units of aprotinin and 10 μM physostigmine. On occasion, 10 μM forskolin was added. When the acetylcholine inhibition of dopamine D_1 receptor-stimulated enzyme activity was assayed, the selective dopamine D_1 receptor agonist (±)-chloro-APB HBr was used at the maximally effective concentration of 1 μM. The concentrations of [α-^{32}P]ATP, GTP and $MgCl_2$ were 0.1, 0.01 and 0.8 mM, respectively. The incubation was started by adding the tissue preparation (30–50 μg of protein) and was carried out at 30°C for 10 min. In some experiments, the tissue was preincubated with muscarinic toxin 3 (at three times the final concentrations) for 15 min at ice-bath temperature. Acetylcholine was then added and the incubation was started by the addition of the reaction mixture. [^{32}P]cyclic AMP was isolated by sequential chromatogra-

phy on Dowex 50 W and alumina columns as described by Salomon et al. (1974).

Protein content was determined by the method of Bradford (1976) with bovine serum albumin used as the standard.

2.4. Statistical analysis

Results are presented as mean ± S.E.M. Agonist concentration–response curves were analyzed by a least squares curve-fitting computer programme (GraphPad Prism, GraphPad Software, San Diego, CA, USA). In experiments where the effect of increasing concentrations of the toxin on the response to a fixed concentration of the agonist was examined, the apparent inhibition constant (K_i) of the toxin was calculated according to the equation (Cheng and Prusoff, 1973):

$$K_i = \frac{IC_{50}}{1 + (A/EC_{50})} \quad (1)$$

where IC_{50} is the concentration of the toxin producing half maximal inhibition; A is the concentration of the agonist and EC_{50} is the agonist concentration producing half-maximal effect. In some experiments, where the effect of a single concentration of the toxin on the agonist concentration–response curve was examined, the K_i was calculated according to the equation:

$$EC_{50b} = EC_{50a}(1 + I/K_i) \quad (2)$$

where EC_{50a} and EC_{50b} are the agonist concentrations producing half-maximal effect in the absence and in the presence of the toxin, respectively, and I is the concentration of the toxin. Statistical significance of the difference between means was determined by Student's t-test.

3. Results

3.1. Effects of muscarinic toxin 3 in olfactory tubercle and corpus striatum

In membranes of rat olfactory tubercle, acetylcholine caused a concentration-dependent inhibition of forskolin-stimulated adenylyl cyclase activity with an EC_{50} value of 0.40 ± 0.03 μM and a maximal effect corresponding to 31.5 ± 3.5% reduction of control activity ($P < 0.001$, $n = 4$). As shown in Fig. 1A, muscarinic toxin 3 completely

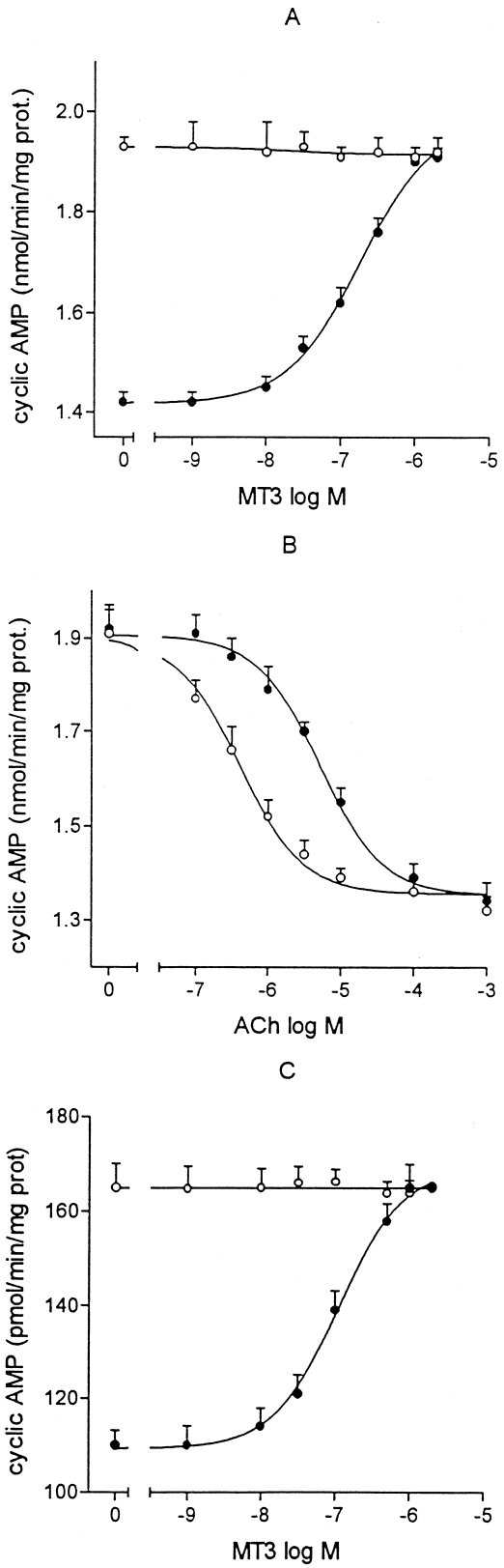


Fig. 1. (A) Effects of muscarinic toxin 3 (MT3) on acetylcholine inhibition of forskolin-stimulated adenylyl cyclase activity in olfactory tubercle. The enzyme activity stimulated by 10 μM forskolin was assayed at the indicated concentrations of muscarinic toxin 3 in the absence (○) and in the presence (●) of 10 μM acetylcholine. Data are the mean ± S.E.M. of three experiments. (B) Concentration-dependent inhibition of forskolin-stimulated adenylyl cyclase activity of olfactory tubercle by acetylcholine (ACh) in the absence (○) and in the presence (●) of 100 nM muscarinic toxin 3. The concentration of forskolin was 10 μM. Data are the mean ± S.E.M. of three experiments. (C) Muscarinic toxin 3 (MT3) antagonism of acetylcholine inhibition of dopamine D_1 receptor-stimulated adenylyl cyclase activity in corpus striatum. The enzyme activity stimulated by 1 μM (±)-chloro-APB HBr was assayed at the indicated concentrations of the toxin in the absence (○) and in the presence (●) of 10 μM acetylcholine. Data are the mean ± S.E.M. of three experiments.

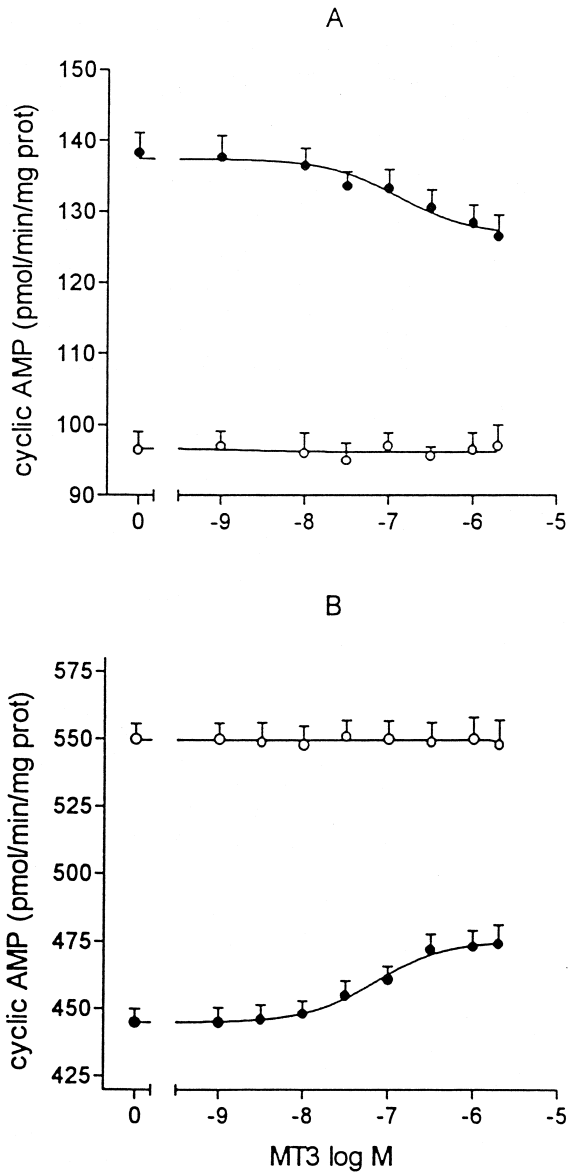
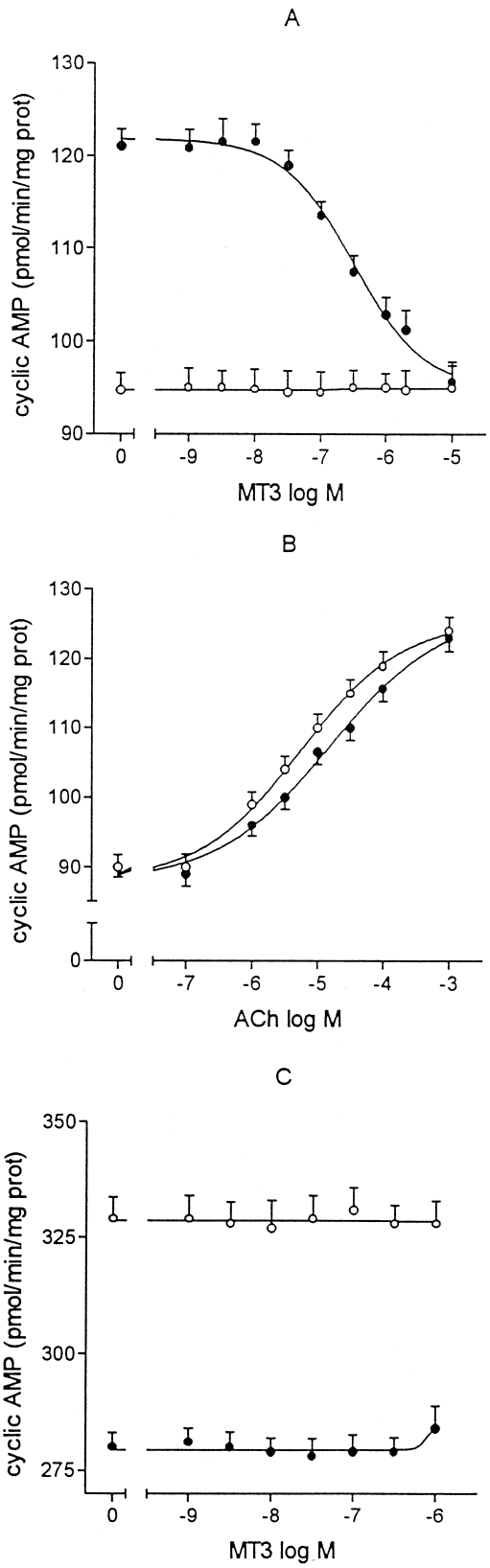


Fig. 2. (A) Effects of muscarinic toxin 3 (MT3) on acetylcholine stimulation of adenylyl cyclase activity in olfactory bulb. The enzyme activity was assayed at the indicated concentrations of the toxin in the absence (○) and in the presence (●) of 5 μM acetylcholine. Data are the mean ± S.E.M. of four experiments. (B) Effects of muscarinic toxin 3 (MT3) on acetylcholine inhibition of forskolin-stimulated adenylyl cyclase activity in rat olfactory bulb. The enzyme activity stimulated by 10 μM forskolin was assayed at the indicated concentrations of the toxin in the absence (○) and in the presence (●) of 5 μM acetylcholine. Data are the mean ± S.E.M. of three experiments.

reversed the inhibition elicited by 10 μM acetylcholine in a concentration dependent manner and with a Hill slope of 1.04 ± 0.08. The K_i value, calculated according to Eq. (1), was 6.8 ± 0.2 nM. In a separate set of experiments, it was found that the addition of 100 nM muscarinic toxin 3 caused a parallel rightward shift in the acetylcholine inhibition curve and increased the agonist EC_{50} value by 12-fold (Fig. 1B). The corresponding K_i value, calculated by Eq. (2), was 8.3 nM.

In rat striatal membranes, acetylcholine inhibited the adenylyl cyclase activity stimulated by the dopamine D_1 receptor agonist (±)-chloro-APB HBr (1 μM) by 34.5 ±



2.5% ($P < 0.001$, $n = 5$) with an EC_{50} of 0.49 ± 0.09 μM. This effect was completely antagonized by muscarinic toxin 3 with a monophasic inhibitory curve (Hill coefficient 1.08 ± 0.07) and a K_i of 4.1 ± 0.5 nM (Fig. 1C).

3.2. Effects of muscarinic toxin 3 in olfactory bulb

As previously reported (Olianas and Onali, 1991), in rat olfactory bulb, acetylcholine stimulated basal adenylyl cyclase activity by 51.2% ($P < 0.001$) with an EC_{50} value of 0.34 ± 0.02 μM. Muscarinic toxin 3, tested at concentrations ranging from 1 nM to 2 μM, maximally reduced the stimulatory effect of acetylcholine by $22.2 \pm 1.9\%$ ($P < 0.05$) with a K_i value of 4.61 ± 0.31 nM (Fig. 2A). Tissue preincubation with the toxin for 15 min did not change either the maximal inhibitory effect or the K_i of the toxin. The adenylyl cyclase activity stimulated by forskolin (10 μM) was significantly inhibited by acetylcholine (25.0% reduction at 10 μM, $P < 0.01$) with an EC_{50} value of 0.45 ± 0.05 μM. Muscarinic toxin 3 maximally antagonized the inhibitory effect of acetylcholine by 30% with a K_i value of 6.60 ± 0.72 nM (Fig. 2B).

3.3. Effects of muscarinic toxin 3 in frontal cortex

We have recently found that in membranes of rat frontal cortex acetylcholine potentiated the stimulation of adenylyl cyclase by CRH (Onali and Olianas, 1996) with an EC_{50} value of 5.0 ± 0.4 μM ($n = 4$). As shown in Fig. 3A, the facilitatory effect of acetylcholine was completely antagonized by increasing concentrations of muscarinic toxin 3 with a K_i of 72.8 ± 3.5 nM and a Hill coefficient of 0.924. The addition of 100 nM toxin increased the agonist EC_{50} value by 2.7-fold (Fig. 3B), yielding a K_i of 58.8 ± 1.8 nM. When forskolin was used to stimulate adenylyl cyclase activity, acetylcholine caused a concentration-dependent inhibitory effect with an EC_{50} of 1.1 ± 0.2 μM. This effect was not antagonized by muscarinic toxin 3 at concentrations up to 1 μM (Fig. 3C). Also in this case, preincubation of the tissue with the toxin for 15 min did not change the effects of the toxin.

Fig. 3. (A) Effects of muscarinic toxin 3 (MT3) on acetylcholine potentiation of CRH-stimulated adenylyl cyclase activity in rat frontal cortex. The enzyme activity stimulated by 0.5 μM CRH was assayed at the indicated concentrations of the toxin in the absence (○) and in the presence (●) of 20 μM acetylcholine. Data are the mean ± S.E.M. of three experiments. (B) Concentration-dependent potentiation of CRH-stimulated adenylyl cyclase activity of frontal cortex by acetylcholine in the absence (○) and in the presence (●) of 100 nM muscarinic toxin 3. The concentration of CRH was 0.5 μM. Data are the mean ± S.E.M. of three experiments. (C) Effects of muscarinic toxin 3 (MT3) on acetylcholine inhibition of forskolin-stimulated adenylyl cyclase activity in frontal cortex. The enzyme activity stimulated by 10 μM forskolin was assayed at the indicated concentrations of the toxin in the absence (○) and in the presence (●) of 20 μM acetylcholine. Data are the mean ± S.E.M. of three experiments.

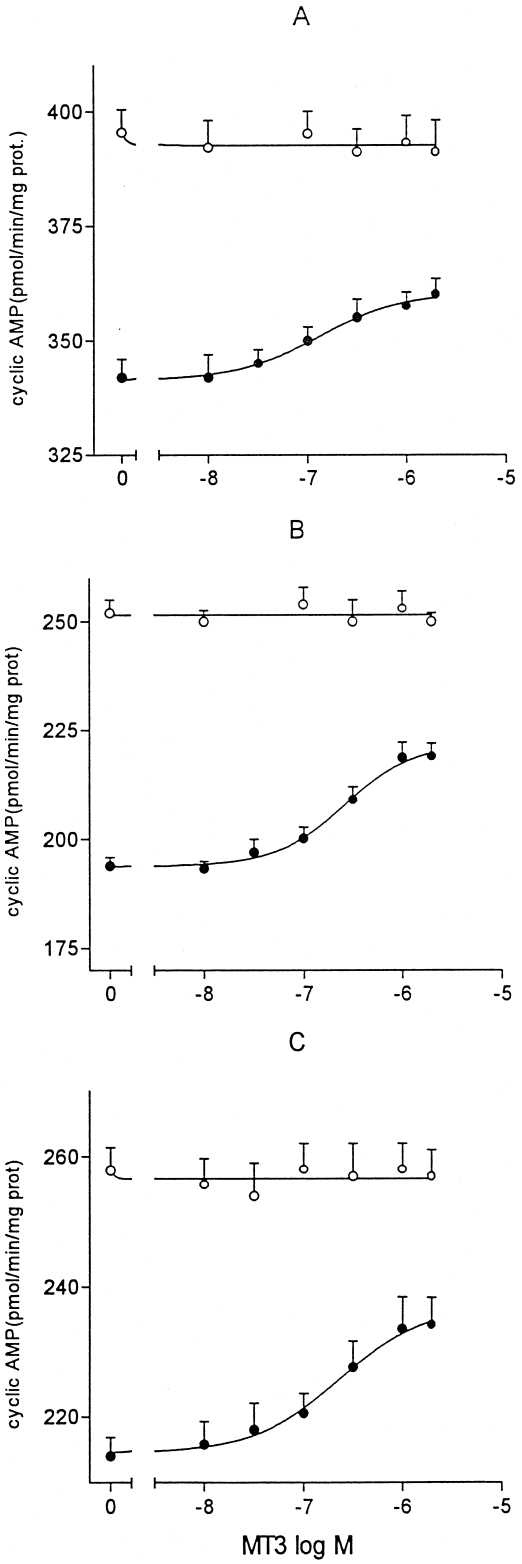


Fig. 4. Effects of muscarinic toxin 3 (MT3) on acetylcholine inhibition of forskolin-stimulated adenylyl cyclase activity in dentate gyrus (A), CA1 (B) and CA2/3 (C) regions of rat hippocampus. The enzyme activity stimulated by 10 μM forskolin was assayed at the indicated concentrations of the toxin in the absence (○) and in the presence (●) of 20 μM acetylcholine. Data are the mean ± S.E.M. of three experiments.

3.4. Effects of muscarinic toxin 3 in hippocampus

In membranes prepared from dentate gyrus, CA1 and CA2/CA3 hippocampal regions, acetylcholine inhibited the forskolin-stimulated adenylyl cyclase activity by 21.0 ± 1.5, 25.2 ± 2.1 and 22.3 ± 1.8% ($P < 0.001$, $n = 3$) with EC_{50} values of 2.0 ± 0.3, 1.4 ± 0.3 and 1.3 ± 0.2 μM, respectively. Muscarinic toxin 3 maximally antagonized the inhibitory effect of acetylcholine (20 μM) by 33 ± 2% with a K_i of 7.0 ± 1.5 nM in dentate gyrus, by 42 ± 4% with a K_i of 6.3 ± 1.0 nM in CA1, and by 48 ± 3.0% with a K_i of 8.5 ± 1.9 nM in CA2/CA3 (Fig. 4).

4. Discussion

Radioligand binding studies using Chinese hamster ovary cells individually expressing the five cloned muscarinic receptor subtypes have shown that muscarinic toxin 3 displays high affinity for the muscarinic M_4 ($pK_i = 8.70$), a lower affinity for the muscarinic M_1 ($pK_i = 7.11$) and a very low affinity ($pK_i < 6.0$) for the muscarinic M_2, M_3 and M_5 receptor subtypes (Jolkkonen et al., 1994). In the present study, the high discriminative property of muscarinic toxin 3 was exploited to dissect out the participation of muscarinic M_4 receptors to the regulation of cyclic AMP formation in various rat brain regions. We found that in the olfactory tubercle, a region which expresses a high density of muscarinic M_4 receptors (Vilaro' et al., 1991; Levey et al., 1991; Yasuda et al., 1993), adenylyl cyclase activity is markedly and potently inhibited by acetylcholine. This response is completely antagonized by muscarinic toxin 3 with a potency ($K_i = 6.8$ nM) that is quite close to the toxin affinity for the cloned muscarinic M_4 receptor. The monophasic inhibition curve displayed by the toxin suggests an interaction with a receptor population of homogenous affinity for the toxin. Thus, in the olfactory tubercle the acetylcholine inhibition of cyclic AMP formation appears to be predominantly mediated by muscarinic M_4 receptors. Similar results were obtained in striatum, another area enriched in muscarinic M_4 receptors (Vilaro' et al., 1991; Levey et al., 1991; Yasuda et al., 1993), where acetylcholine is known to exert an inhibitory effect on the adenylyl cyclase stimulation elicited by dopamine D_1 receptor activation (Olianas et al., 1983). In this brain region, muscarinic toxin 3 caused a complete and monophasic reversal of the acetylcholine inhibitory effect with a high potency ($K_i = 4.1$ nM). These data confirm previous pharmacological investigations using classical antagonists indicating that in rat striatum the muscarinic inhibition of dopamine D_1 receptor activity is mediated by muscarinic M_4 receptors (Olianas and Onali, 1996).

Rat olfactory bulb has been shown to express mRNAs encoding different muscarinic receptor subtypes (Buckley et al., 1988). In this region, activation of muscarinic receptors causes stimulation of basal and neurotransmitter-stimulated adenylyl cyclase activities and inhibition of forskolin and Ca^{2+}/calmodulin-stimulated enzyme activities (Olianas and Onali, 1994). This bimodal control of cyclic AMP formation likely involves the coupling of muscarinic receptors to different molecular forms of adenylyl cyclase (Onali and Olianas, 1995). Pharmacological analysis using a number of classical receptor subtype-preferring antagonists indicated that a heterogeneous receptor population participated in the stimulation of adenylyl cyclase activity, the predominant receptor possibly being pharmacologically equivalent to the m4 gene product (Olianas and Onali, 1991). The present study, however, shows that only a limited fraction (~ 25%) of the acetylcholine stimulatory response is antagonized by muscarinic toxin 3 with high affinity and therefore can be attributed to muscarinic M_4 receptors. Similar results are obtained for the muscarinic inhibition of forskolin-stimulated enzyme activity. For both responses, the remaining fractions appear insensitive to toxin concentrations above 1 μM and may be mediated by muscarinic M_2 receptors.

Recent investigations in rat frontal cortical membranes have detected the presence of muscarinic receptors that potentiate CRH- stimulated adenylyl cyclase activity (Onali and Olianas, 1996). These receptors were blocked with high affinity by pirenzepine and telenzepine, suggesting the involvement of the muscarinic M_1 receptor subtype. The present study shows that muscarinic toxin 3 antagonizes the acetylcholine potentiation of CRH-stimulated adenylyl cyclase activity with a K_i value (58.8 nM) close to the affinity for the cloned muscarinic M_1 receptor ($pK_i = 7.11$; Jolkkonen et al., 1994) and to the potency in inhibiting the muscarinic stimulation of phosphoinositide hydrolysis in cerebral cortex ($K_i = 113$ nM; Olianas et al., 1996), a response predominantly mediated by muscarinic M_1 receptors (Forray and El-Fakahany, 1990). These data support the classification of the cortical muscarinic receptor facilitating CRH transmission as M_1. Pharmacological studies have previously indicated that in rat cortical minces forskolin-stimulated cyclic AMP accumulation was inhibited by muscarinic M_4 receptor activation (McKinney et al., 1991). Accordingly, in the present study acetylcholine is found to inhibit forskolin-stimulated adenylyl cyclase of frontal cortex. However, the effect is not antagonized by muscarinic toxin 3 at concentrations highly effective on muscarinic M_4 receptors. Thus, it appears that, at least in frontal cortex, the acetylcholine inhibition of forskolin-stimulated cyclic AMP formation is mediated by muscarinic M_2, rather than M_4, receptors.

Although cyclic AMP plays an important role in the regulation of short and long term synaptic transmission in the hippocampus (Chaver-Noriega and Stevens, 1994; Weisskopf et al., 1994), little information is available on the pharmacological properties of muscarinic receptors regulating adenylyl cyclase activity in this brain area. As recent immunological studies have shown a distinct pattern

of distribution of each muscarinic receptor subtype within hippocampal structures (Levey et al., 1995), we have analyzed the effect of acetylcholine on the forskolin-stimulated adenylyl cyclase activity in microdissected tissue of dentate gyrus, CA1 and CA2/CA3 hippocampal regions. Acetylcholine is found to exert an inhibitory effect in all the three regions with similar potencies and efficacies. In each region examined, muscarinic toxin 3 reverts with high affinity (K_i values = 6–8 nM) a fraction of the acetylcholine inhibitory effect, whereas a major portion of the response is not affected by high toxin concentrations. These data indicate that both muscarinic M_4 and M_2 receptors mediate the acetylcholine inhibition. The finding that in CA1 and CA2/CA3 regions the fraction of the cyclase response sensitive to muscarinic toxin 3 is higher than in dentate gyrus (42 and 48 vs. 33%, respectively) may reflect either a differential distribution of muscarinic M_4 receptor-adenylyl cyclase complexes or a variability in the receptor coupling efficiency.

In conclusion, the present study demonstrates that muscarinic toxin 3 is highly capable of discriminating among muscarinic receptors regulating cyclic AMP formation in different brain regions and allows for the first time a reliable titration of the contribution by the muscarinic M_4 receptor subtype.

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