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ORIGINAL PAPER

Muscarinic Inhibition of Hippocampal and Striatal Adenylyl Cyclase is Mainly Due to the M_4 Receptor

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Abstract The five muscarinic acetylcholine receptors (M_1–M_5) are differentially expressed in the brain. M_2 and M_4 are coupled to inhibition of stimulated adenylyl cyclase, while M_1, M_3 and M_5 are mainly coupled to the phosphoinositide pathway. We studied the muscarinic receptor regulation of adenylyl cyclase activity in the rat hippocampus, compared to the striatum and amygdala. Basal and forskolin-stimulated adenylyl cyclase activity was higher in the striatum but the muscarinic inhibition was much lower. Highly selective muscarinic toxins MT1 and MT2—affinity order $M_1 \geq M_4 >>$ others—and MT3—highly selective M_4 antagonist—did not show significant effects on basal or forskolin-stimulated cyclic AMP production but, like scopolamine, counteracted oxotremorine inhibition. Since MTs have negligible affinity for M_2, M_4 would be the main subtype responsible for muscarinic inhibition of forskolin-stimulated enzyme. Dopamine stimulated a small fraction of the enzyme (3.1% in striatum, 1.3% in the hippocampus). Since MT3 fully blocked muscarinic inhibition of dopamine-stimulated enzyme, M_4 receptor would be responsible for this regulation.

Keywords Muscarinic acetylcholine receptor · Adenylyl cyclase · Muscarinic toxins · Hippocampus · Striatum

Introduction

The modulation of excitatory transmission by muscarinic acetylcholine receptors (mAChRs) seems particularly relevant to learning and memory processing in the hippocampal formation, where the diversity and differential localization of the receptors are likely to account for the complex cholinergic modulation in this structure [1, 2]. The non-selective muscarinic antagonist scopolamine causes amnesia for many behavioral tasks in rats when directly administered into the hippocampus immediately after training [3], supporting the idea of the involvement of hippocampal mAChRs in memory consolidation.

There are five subtypes of mAChRs (M_1–M_5); all of them are expressed in the brain with differential localization and more than one subtype is often expressed in the same cell [4].

It was previously reported that in the hippocampus non-M_4 receptors mainly involving the M_1 subtype, would amount around 60%, whereas the M_4 subtype would be about 24%. In the striatum M_1 would amount about 57% and M_4 27%, and about 54 and 15%, respectively, in the

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amygdala [5]. There is a conspicuous expression of mAChR in both hippocampus and striatum, where many interneurons are cholinergic, compared to the amygdala. In spite of the striatal origin of many interneurons in this structure, most of them are GABAergic [2, 6]. It is interesting to note that pyramidal neurons, granule cells and interneurons in the hippocampal formation are immuno-positive for M_1 and M_4 receptors, while exhibiting M_2 weak staining [7].

The role of discrete receptor subtypes in vivo has been poorly understood due to the lack of pharmacological tools selective enough to discriminate between them. M_1, M_3 and M_5 subtypes participate in intracellular signaling mainly by coupling to the phosphoinositide pathway through $G_{q/11}$ proteins, while M_2 and M_4 receptors are preferentially coupled to the inhibition of stimulated adenylyl cyclase through $G_{i/0}$ [8, 9]. In addition, there are evidence that M_1 and M_3 could modulate cyclic AMP production in reconstituted systems in transfected cell lines [10, 11]. Most of the previous studies were carried out with antagonists of limited selectivity for receptor subtypes; hence, none of them allowed a clear discrimination between M_2 and M_4 receptors. Mistry and co-workers [12] have shown that at both M_2 and M_4 receptors, various agonists tested were more potent in mediating $G_{i/0}$ versus G_s-coupled responses and that there were no significant differences for the agonist oxotremorine at inhibiting cyclic AMP production. Most of the literature reports the effect of agonists on cloned receptors expressed in cell lines and there are few reports on native receptors.

Muscarinic toxins (MTs) are small proteins in *Dendroaspis* snake venom that show very high selectivity for some mAChR subtypes and have been used both in vitro and in vivo [13–15]. MT1 and MT2 selectively bind to M_1 and M_4 receptor subtypes; MT1 shows similar affinities for both receptors, while MT2 has a 4-fold higher affinity for M_1 than for M_4. In pharmacological assays, MT1 behaved as an M_1 agonist in isolated nerve-muscle preparations [13, 15, 16]. In previous assays it was shown that MT2 enhanced carbachol-induced phosphatidylinositol turnover in homogenates of rat cerebral cortex [17]. Both MT1 and MT2 caused facilitation of memory consolidation when injected immediately after training into the rat hippocampus; this effect was attributed to their agonist activity at M_1 receptors [18].

On the other hand, another toxin, MT3, exhibits 214-fold higher affinity for M_4 than for M_1 receptors [19]. When injected into the dorsal hippocampus of rats immediately after training in an inhibitory avoidance task, MT3 caused amnesia on the retrieval tested 24 h later, whereas both MT1 and MT2 were facilitatory [19, 20].

All three toxins show negligible binding to muscarinic receptor subtypes other than M_1 and M_4; in particular, they do not bind M_2 receptors [21].

It has been previously reported that MT3 antagonized the acetylcholine inhibition of adenylyl cyclase activity in certain brain regions [22]. Both in the rat olfactory tubercle and the striatum, MT3 appeared to completely antagonize the acetylcholine induced inhibition of adenylyl cyclase, stimulated either by forskolin in the olfactory tubercle or by a D1 dopamine agonist in the striatum. In contrast, in both the frontal cortex and the hippocampus the cholinergic inhibition appeared to be only partially reversed (about 40%) by MT3 [22].

Although the binding properties of both MT1 and MT2 to M_1 and M_4 receptors and their agonist action at M_1 are already known, there is no previous report on the effect of these toxins on cyclic AMP production, nor on their agonist/antagonist actions at M_4 receptors either. Therefore, we intended to clarify this point.

The main goal in the present study was to shed more light on the regulation of adenylyl cyclase activity by mAChR subtypes in the hippocampus. Taking into account the participation of the hippocampal cholinergic muscarinic transmission in learning and memory [1] and the reported effects of MTs on memory in rats [13, 19, 20], this work would also contribute to further understand the participation of mAChR subtypes in cognitive functions.

The amygdala is relevant for fear-related learning and muscarinic influences are essential for the amygdala-mediated modulation of memory [23]. In the striatum, the cholinergic interneurons are relevant to circuits that participate in motor control [24]. The mAChR are conspicuously expressed in both hippocampus and striatum, while they are relatively scarce in the amygdala [2, 6].

We have studied the action of muscarinic agonists and antagonists on adenylyl cyclase activity stimulated either by forskolin or dopamine in the hippocampal formation, compared to the striatum and amygdala of the rat, and we have determined the effects of the subtype selective compounds MT1, 2 and 3.

Experimental Procedure

Materials

Muscarinic toxins were purified from *Dendroaspis angusticeps* snake venom (J. Leakey Ltd., Kenya, East Africa) [25]. Forskolin (7-deacetyl-7-(*O*-*N*-methylpiperazine)-γ-butyryl-) dihydrochloride was from Calbiochem-Novabiochem Corporation (La Jolla, CA, USA). [α-^{32}P]-ATP (3000 Ci mmol^{-1}) and [2,8-^{3}H]-cyclic AMP (31.7 Ci mmol^{-1}) were purchased from Dupont-New England Nuclear (Boston, MA, USA). Oxotremorine sesquifumarate, scopolamine methylbromide and other reagents were from Sigma (St Louis, MO, USA).

Experiments with rats were performed in strict accordance to the Review Committee of the Veterinary School, University of Buenos Aires, the Brazilian law to the recommendations of the Brazilian Society for Neurosciences, and the International Brain Research Organization (IBRO), and are in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (publication No 85-23, revised 1985).

Tissue Dissection and Membrane Preparation for Adenylyl Cyclase Assays

Male Wistar rats (180–250 g) were killed by decapitation, their brains were removed, washed twice in 0–4°C 0.32 M sucrose, and hippocampi, striata and amigdalae were dissected.

The structures were homogenized in 10 volumes (w/v) of ice-cold hypotonic buffer: 10 mM *N*-[2-hydroxyethyl] piperazine-*N*′-[2-ethanesulfonic acid] (HEPES)/NaOH, 0.3 mM ethylene glycol-bis(ß-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 2.3 mM $MgCl_2$, pH 7.4, plus 0.32 M sucrose; the homogenates were centrifuged for 10 min at 1,000×*g* at 4°C. The supernatants were centrifuged at 11,000×*g* for 20 min at 4°C. The pellets were resuspended, incubated for 20 min in 20 ml of hypotonic buffer, and centrifuged at 27,000×*g* for 20 min at 4°C. The final pellets were resuspended in the incubation buffer: 50 mM HEPES/NaOH, 0.3 mM EGTA, 0.1 mM guanosine 5′-triphosphate (GTP), 2.3 mM $MgCl_2$, 2 mM 3-isobutyl-1-methylxanthine, 0.2 mM adenosine 5′-triphosphate (ATP), 5 mM phosphocreatine, 50 U/ml creatine phosphokinase, 0.5 mg/ml bovine serum albumin, 0.1 mg/ml bacitracin, 25 µg/ml aprotinin, pH 7.4, and were left for 20 min on ice.

Adenylyl Cyclase Assays

Adenylyl cyclase assays were performed according to Olianas and co-workers [26], with slight modifications. The enzyme activity was measured in 100 µl of incubation buffer containing 50–100 cpm/pmol [α-^{32}P]-ATP and each of the several agonists and antagonists used. The reaction was started by addition of 30 µl (30–40 µg protein) of membrane preparation, immediately incubated at 35°C for 10 min, and stopped with 100 µl of 40 mM ATP, 2% sodium dodecyl sulfate, pH 7.5. To estimate the adenosine 3′:5′-cyclic monophosphate (cyclic AMP) recovery, 0.2 µCi [2,8-^{3}H]-cyclic AMP were added and the volume was adjusted to 1 ml with water.

The cyclic AMP produced during the reaction was isolated by sequential chromatography on Dowex and alumina columns according to Salomon et al. [27], with slight modifications. The samples were run in Dowex 50WX8 columns; the first elution volume plus two rinses of 1 ml were discarded. Then, 3 ml of water were added. The eluate was collected and 200 µl of 1.5 M imidazole buffer, pH 7.2, were added; it was run through the alumina column and the eluate was collected. Once the elution has finished, the columns were washed with 1 ml of 0.1 M imidazole buffer, pH 7.5.

The eluates were collected in glass vials and mixed with 16 ml of liquid scintillation cocktail Optiphase HiSafe 2 (Wallack) in order to measure the radioactivity.

Curve Fitting

Data were analysed by non-linear regression using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA, USA, www.graphpad.com). Equations for either one site or two sites—sigmoidal dose–response curves—were fitted to data from enzyme activity assays; the software was used to compare the results to find out the best regression according to an F-test, by balancing the change in sum of squares and the degrees of freedom for each experimental data-set. For the two-sites regression model, two percentages were calculated, one for the proportion of high affinity sites and the other for the low affinity sites; and two EC_{50} values were also calculated, one for high and another for low affinity populations.

Results

Muscarinic Action on Forskolin-stimulated Cyclic-AMP Production

Hippocampal adenylyl cyclase activity was stimulated by forskolin in a dose-dependent manner and fitted to a sigmoidal dose–response curve, with a maximal stimulation reached at 0.5 mM forskolin (Fig. 1a). At 100 µM forskolin, the percentage of stimulated adenylyl cyclase activity in hippocampal membranes was 978.4 ± 102.3% ($n = 38$) with respect to the basal level of cyclic AMP produced in the absence of forskolin. The cyclic AMP production are shown in Table 1; the basal level in the hippocampus was 3.8 ± 0.9 nmol min^{-1} mg protein^{-1}.

In the striatum, the basal cyclic AMP production was 33.0 ± 1.2 nmol min^{-1} mg protein^{-1} (Table 1). With 100 µM forskolin adenylyl cyclase activity increased to 2281.0 ± 124.4% ($n = 42$) respect to the basal level (Fig. 1b). As expected, the stimulation increased with forskolin concentration, but the enzyme activity did not reach a plateau at the same forskolin concentrations it did in the hippocampus. Since 100 µM forskolin produced a significant stimulation in both structures without reaching

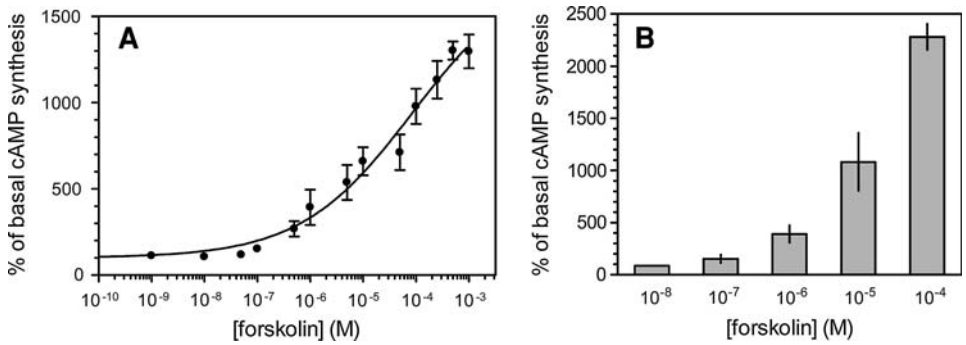

Fig. 1 Dose–response curve of stimulation of adenylyl cyclase by forskolin in rat hippocampal membranes (**a**) and bar diagram representing the forskolin stimulation in striatal membranes (**b**). Data are represented as mean ± SEM (as percentages). 100% corresponds to [^{32}P]-cyclic AMP produced in basal conditions, without forskolin. Basal production of [^{32}P]-cyclic AMP was 3.8 ± 0.9 nmol min^{-1} mg protein^{-1} ($n = 6$) for the hippocampus and 33.0 ± 1.2 nmol · min^{-1} mg protein^{-1} ($n = 6$) for the striatum

Table 1 Effects of forskolin and dopamine on [^{32}P]-cyclic AMP production in hippocampal, striatal and amygdala membranes

	Hippocampus	Striatum	Amygdala
Basal	3.8 ± 0.9	33.0 ± 1.2	3.3 ± 0.8
Forskolin			
10^{-7} M	5.8 ± 0.5	50.6 ± 13.2	–
10^{-5} M	25.1 ± 3.8	129.1 ± 27.4	13.4 ± 1.6
10^{-4} M	37.2 ± 3.9	752.7 ± 40.9	22.0 ± 2.4
Dopamine			
10^{-6} M	4.0 ± 0.1	33.1 ± 3.7	–
10^{-4} M	4.1 ± 0.1	48.5 ± 2.9	–
10^{-3} M	4.4 ± 0.2	54.9	–

Values in nmol of [^{32}P]-cyclic AMP min^{-1} mg protein^{-1} (media ± SEM; except dopamine 10^{-3} M in striatum, with no replicates)

saturation levels, we chose this concentration for the following assays with muscarinic toxins MT1, MT2 and MT3.

Hippocampal membranes stimulated by 100 μM forskolin (considered as 100%) showed a 110.7 ± 9.5% ($n = 13$) cyclic AMP production in the presence of 10 μM MT1, while in the striatum it was 96.4 ± 8.8% ($n = 6$). Therefore, MT1 does not significantly modify forskolin-stimulated cyclic AMP production under these conditions.

The addition of 10 μM MT3 to striatal membranes did not produce statistically significant difference in basal adenylyl cyclase activity (97.2 ± 3.9%, $n = 5$). Likewise, MT3 up to 25 μM did not produce any significant change in the basal level of enzyme activity (99.8 ± 2.4%, $n = 2$) and did not significantly affect the forskolin stimulated activity in hippocampal synaptosomal membranes (10 μM MT3: 118.9 ± 15.5%, $n = 11$).

Figure 2 corresponds to the inhibition curves of cyclic AMP synthesis—forskolin induced—by the specific muscarinic agonist oxotremorine in hippocampal synaptosomal membranes, either with or without the antagonist scopolamine (Fig. 2a) or the different muscarinic toxins (Fig. 2b–d). The agonist oxotremorine caused a biphasic inhibition on forskolin-stimulated adenylyl cyclase activity. The curves were better fitted to a two-step, rather than a one-step transition equation. In general, the inhibition was apparent at 10^{-8} M, and the first step follows up to 10^{-5} M. In the hippocampus, this inhibition by the lower concentrations of oxotremorine (corresponding to "high-affinity" inhibition) amounted 13.5 ± 2.8% of the total forskolin-stimulated adenylyl cyclase activity, with an IC_{50} of 2.3 nM. This first step of the curve was fully blocked by 10 μM scopolamine, a rather specific muscarinic antagonist, indicating that this was actually mediated by muscarinic receptors (Fig. 2a). The second step, a steeper decrease in activity corresponding to the "low-affinity" inhibition fraction, was obtained with oxotremorine concentrations higher than 10^{-5} M. The dose-dependent portion of the curve corresponding to this low-affinity step reached a 44.7 ± 4.4% further inhibition of forskolin-stimulated cyclic AMP production. In contrast to the first part of the curve, this inhibition was not affected by scopolamine (Fig. 2a).

Both MT1 and MT3 were used in concentrations much higher than their respective K_i at M_4 receptors (126 nM for MT1 and 1.2 nM for MT3) [19, 21] to investigate their effects on the inhibition caused by oxotremorine. Scopolamine, as well as both toxins MT1 (1 μM) (Fig. 2b) and MT3 (100 nM) (Fig. 2d) eliminated the high-affinity inhibition by oxotremorine, showing similar monophasic curves, without affecting the low-affinity inhibition step (where the estimated IC_{50} value for oxotremorine was about 1 mM). As can be seen in Fig. 2c, the curve for oxotremorine inhibition in the presence of MT2 (1 μM) showed a slightly different profile: there was a rightward shift of the high-affinity portion of the curve. This concentration of the toxin did not significantly affect forskolin-stimulated cyclic AMP production (100.9 ± 4.5%). Hence, the MTs have counteracted the inhibition caused by low

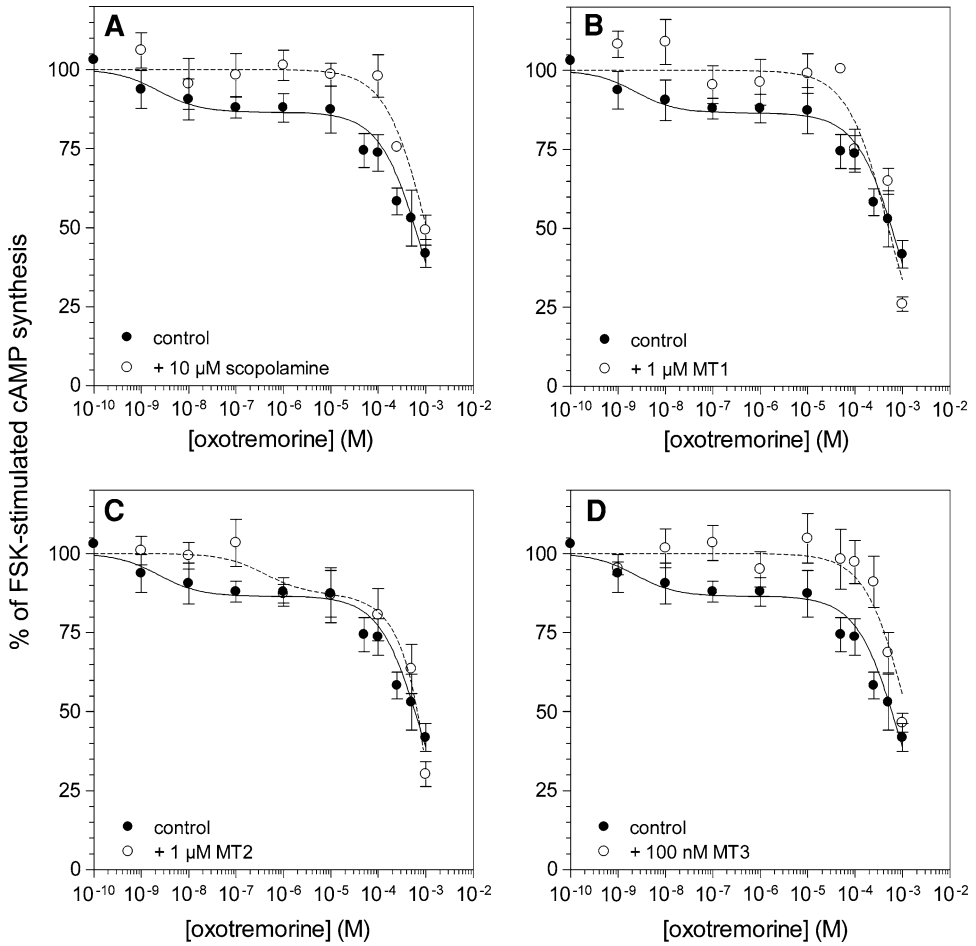

Fig. 2 Inhibition curves by oxotremorine, of adenylyl cyclase activity stimulated by forskolin (100 μM) in hippocampal membranes, either in the presence (○) or absence (●) of **a** 10 μM scopolamine; **b** 1 μM MT1; **c** 1 μM MT2; **d** 100 nM MT3. Data are represented as mean ± SEM (as percentages). 100% corresponds to [^{32}P]-cyclic AMP produced in the presence of 100 μM forskolin. The inhibition curves by oxotremorine alone are better fitted to a two-step transition equation. Inhibition by low concentrations of oxotremorine amounted 13.5% of total forskolin-stimulated adenylyl cyclase activity, IC_{50} of 2.3 nM. The inhibition curves in the presence of 10 μM scopolamine (**a**), 1 μM MT1 (**b**) and 100 nM MT3 (**d**) were monophasic, showing only the oxotremorine low-affinity inhibition, with IC_{50} of 1.0, 0.5 and 1.2 mM respectively ($n = 3$–10). The inhibition curve in the presence of 1 μM of MT2 was biphasic (**c**), with a high-affinity inhibition by oxotremorine of 13.5%, $IC_{50\ 1}$ of 402.8 nM and $IC_{50\ 2}$ 16.1 mM

concentrations of the muscarinic agonist oxotremorine (high-affinity) upon forskolin-stimulated adenylyl cyclase activity. However, neither scopolamine nor the MTs were able to block the inhibition caused by oxotremorine at concentrations higher than 10^{-5} M.

In assays carried out in striatal membranes, the inhibition of the forskolin-stimulated cyclic AMP production by oxotremorine showed dose–response curves rather similar to those found in the hippocampal membranes and which were also well fitted by a two site model. However, the high-affinity inhibition by oxotremorine (10^{-8}–10^{-4} M) amounted only 6.8 ± 4.2% of the total forskolin-stimulated adenylyl cyclase activity with an IC_{50} of about 0.3 nM; this decrease was not statistically significant. The second step, a steeper decrease corresponding to the low-affinity inhibition fraction obtained with oxotremorine concentrations between 10^{-4} and 10^{-3} M, reached a 34.5 ± 8.2% further inhibition of forskolin-stimulated cyclic AMP production. This latest inhibition was not modified by scopolamine either. Like in the hippocampal membranes, 1 μM MT1 as well as scopolamine, were not able to reverse the oxotremorine inhibition at agonist concentrations higher than 10^{-4} M.

In amygdala membranes, the stimulation of adenylyl cyclase activity by 100 μM forskolin reached about 670% with respect to the basal activity (3.3 ± 0.8 nmol of [^{32}P]-cyclic AMP min^{-1} mg protein^{-1}, Table 1). But in this structure, the muscarinic agonist oxotremorine up to 10^{-4} M did not appear to inhibit the forskolin-stimulated adenylyl cyclase activity.

Muscarinic Action on Dopamine-stimulated Cyclic-AMP Production

The dose–response curves for the stimulation of cyclic AMP production by dopamine in hippocampal and striatal synaptosomal membranes are shown in Fig. 3. The increase in cyclic AMP production stimulated by 1 mM dopamine corresponds to 166% in the striatum and to 116% in the hippocampus. As shown in Table 1, cyclic AMP production stimulated by dopamine in the striatum was about 12-fold that in the hippocampus. As we could not performed reliable assays in the hippocampus due to the low increase reached by dopamine stimulation, we were only able to study the muscarinic effects on dopamine stimulation on striatal tissue.

The inhibition by oxotremorine of dopamine-stimulated adenylyl cyclase in the striatum is represented by the bar diagram in Fig. 4. The adenylyl cyclase activity stimulated by 100 μM dopamine reached 140.9 ± 5.0% of the basal level. This stimulated activity was inhibited to 121.2 ± 3.9% by 10 μM oxotremorine. Thus, the agonist was able to inhibit up to 48% of the activity increase by 100 μM dopamine in the striatum. This inhibition by 10 μM oxotremorine was completely blocked by 10 μM MT3 (144.2 ± 5.4%, Fig. 4).

On the other hand, we did not find any significant muscarinic effect on adenylyl cyclase activity stimulated by dopamine in amygdala membranes.

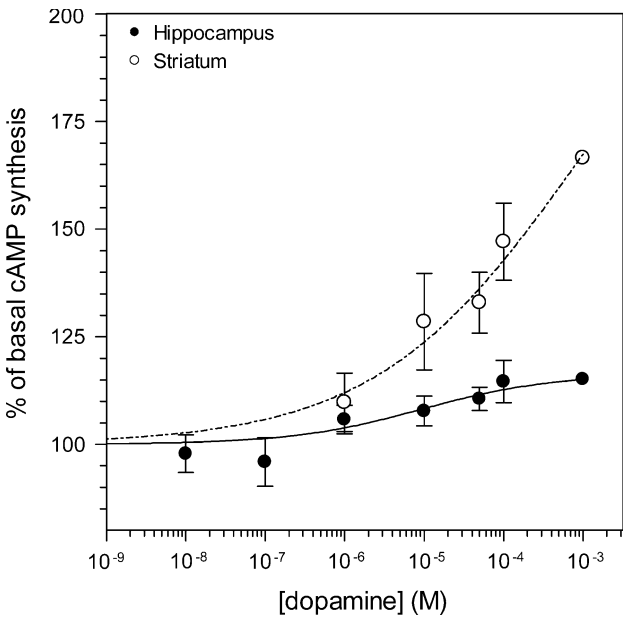

Fig. 3 Dose–response curve of stimulation of adenylyl cyclase by dopamine in rat hippocampal (●) and striatal (○) membranes. Data are represented as mean ± SEM (as percentages). 100% corresponds to [^{32}P]-cyclic AMP produced in basal conditions, without forskolin. Basal production of [^{32}P]-cyclic AMP was 3.8 ± 0.9 nmol min^{-1} mg protein^{-1} for hippocampal membranes ($n = 6$) and 33.0 ± 1.2 nmol min^{-1} mg protein^{-1} for striatal membranes ($n = 6$)

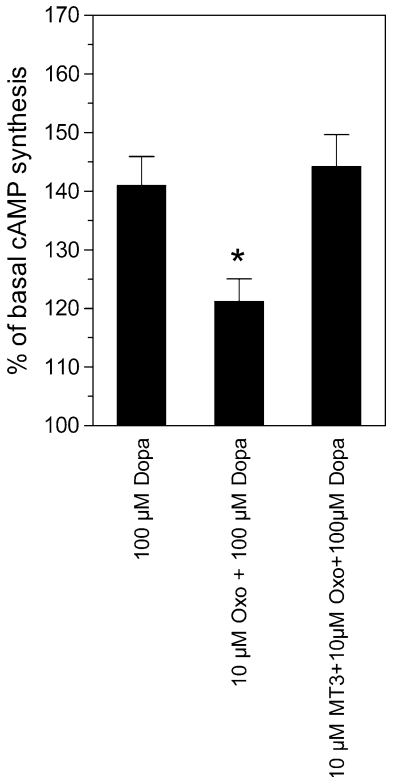

Fig. 4 Bar diagram representing the effect on striatal membranes of oxotremorine (Oxo) and MT3 over [^{32}P]-cyclic AMP produced under 100 μM dopamine (Dopa) stimulation. Data are represented as mean ± SEM (as percentages). 100% corresponds to [^{32}P]-cyclic AMP produced in basal conditions, without dopamine. Basal production was 33.0 ± 1.2 nmol min^{-1} mg protein^{-1} ($n = 6$). *Mean ± SEM was significantly different compared to the cyclic AMP production in the presence of 100 μM dopamine alone ($P < 0.05$, ANOVA followed by Dunnet′s multiple comparison test)

Discussion

In this paper we report results of experiments carried out in rat synaptosomal membranes of rat hippocampus, striatum and amygdala. The basal activity of adenylyl cyclase in the rat striatum membranes was about 8.7 fold of the activity in the hippocampus and 10-fold that in the amygdala. The stimulation of synaptosomal membranes from striatum by forskolin was about 20-fold that in the hippocampus and 34-fold that in the amygdala. It was shown that in both the striatum and hippocampus the enzyme activity increased with forskolin concentration but did not reach a steady state in striatum at the same concentrations it did in the hippocampus (Fig. 1; Table 1).

It is widely accepted that M_2 and M_4 [8] mAChR preferentially interact with $G_{i/0}$ proteins causing inhibition of previously stimulated adenylyl cyclase [28]. If this were the case in the hippocampus, both M_2 and M_4 receptor subtypes should be recruited for the inhibition of adenylyl cyclase by the relatively non-selective agonist oxotremorine.

Here we show that the inhibition of adenylyl cyclase by oxotremorine in the hippocampus appeared biphasic, with both high-affinity and low-affinity inhibition steps (Fig. 2). The first part of the agonist inhibition curve was fully blocked by the non-selective muscarinic antagonist scopolamine, which did not affect the second low-affinity step. Therefore, these results are consistent with the muscarinic modulation of adenylyl cyclase in the hippocampus being mainly—if not only—represented by the high-affinity oxotremorine inhibition. Although some minor muscarinic contribution to the low-affinity oxotremorine inhibition can not be completely discarded, it did not appear significant in this case, since there was not any detectable antagonism by scopolamine. Hence, this also corroborates that the muscarinic inhibition affected just a small portion of all the forskolin-stimulated adenylyl cyclase in the hippocampus (13.5%, Fig. 2). Of course, there are more adenylyl cyclase variants which are targets for forskolin stimulation—i.e., different isoforms, different location [29]—than those regulated by mAChR.

The muscarinic toxins used did not produce significant direct effects on either basal or forskolin-stimulated adenylyl cyclase activity. MT2 has lower affinity than MT1 at both M_1 and M_4 receptors, but discriminates better between them (4-fold higher affinity for M_1 than for M_4; 21). MT3 is highly selective for M_4 receptors [19, 30]. None of the muscarinic toxins used have any detectable binding to M_2 receptors [14, 19, 21].

The full blockade of the muscarinic inhibition of hippocampal adenylyl cyclase by both MT1 and MT3 (Fig. 2b, d) was rather similar to that by scopolamine (Fig. 2a). On the other side, the slightly different effect of MT2—a rightward shift—on the high-affinity oxotremorine inhibition of hippocampal adenylyl cyclase might be due to the lower affinity this toxin exhibits at M_4 receptor, compared to the other muscarinic toxins used (Fig. 2c). Hence, the muscarinic toxins antagonism at M_4 receptors would counteract the oxotremorine inhibition of stimulated adenylyl cyclase. Both MT1 and MT2 acted as M_1 agonists in several physiological paradigms, including central and peripheral nervous system [13, 15, 18, 21, 31] and in cell lines [16, 32]. In our assays, MT1, MT2 and MT3 behaved as M_4 antagonists, in agreement with the reported data by Olianas et al. [22]. MT3 was reported to also behave as an M_4 antagonist at the rabbit anococcygeus muscle preparation [16] and in memory consolidation in the rat [19].

It had previously been suggested that neither M_1 nor M_2 receptors were responsible for cholinergic inhibition of adenylyl cyclase in the striatum [33] or in the cerebral cortex of the rat [34], whereas adenylyl cyclase muscarinic inhibition would be mediated by M_2 receptors in the brain stem [35].

Oxotremorine inhibited the forskolin-stimulated adenylyl cyclase in the striatum (though not significantly in low concentrations) following a biphasic inhibition curve, as it did in the hippocampus. Again, the oxotremorine low-affinity inhibition step was not blocked by scopolamine. In contrast, it has been reported that cyclic AMP production stimulated by forskolin (10 μM) was inhibited to around 25% by acetylcholine and 24% by carbachol in the rat striatum following a monophasic curve [36].

According to our estimations from the data reported by Olianas et al. [22], 10 μM forskolin stimulated cyclic AMP synthesis to around 390 pmol mg^{-1} min^{-1} in dentate gyrus, and to approximately 255 pmol mg^{-1} min^{-1} in CA regions of rat hippocampal formation. These values are much lower than the data here reported in Table 1. It is difficult to interpret why the results are so different, but the discrepancies could be due to the different membrane preparation. These authors reported that acetylcholine (20 μM) inhibited forskolin stimulation of adenylyl cyclase by 21–25% in the hippocampal regions, and that the toxin MT3 antagonized this effect of acetylcholine by partially reversing that inhibition to about 33–48%. Although we were not able to calculate from the published data the ratio of adenylyl cyclase stimulated respect to the basal level, the percentages of reversion of the inhibition by 20 μM acetylcholine would be comparable to the inhibition we obtained with lower oxotremorine concentrations. However, in our assays MT3 did fully block only that adenylyl cyclase inhibition which was also antagonized by scopolamine.

As it was already mentioned, neither MT3 nor scopolamine did fully block the oxotremorine inhibition, but they acted in a similar way, reversing a similar proportion of that inhibition.

It was previously reported that acetylcholine inhibited the forskolin-stimulated adenylyl cyclase in the rat hippocampus with much higher EC_{50} than those we found with oxotremorine and that MT3 blocked just a fraction of that inhibition, whilst most of the response was not affected [22]. Thus, it was suggested that M_2 receptors were also involved in this regulation. In contrast, we have shown that the high-affinity inhibition by oxotremorine, the only that can be considered as pure muscarinic since it was blocked by scopolamine, was also fully blocked by muscarinic toxins in the hippocampus. Therefore, we strongly suggest that M_4 receptors are responsible for either all or most of the muscarinic inhibition of forskolin-stimulated adenylyl cyclase in hippocampus. The discrepancy may be due to the fraction of adenylyl cyclase inhibition that was considered as modulated by muscarinic receptors. We believe that part of the inhibition by either high oxotremorine concentration or by other agonists should not be consider as muscarinic since it was caused by very high agonist concentrations and was not blocked by scopolamine;

therefore, it should not be expected to be antagonized by any of the toxins.

Olianas et al. [26, 37] reported that acetylcholine inhibited the basal level of striatal adenylyl cyclase activity as well as that stimulated either by forskolin or by a D1 receptor agonist. It was reported that carbachol also caused an inhibition in basal cyclic AMP production [38]. Since this inhibition was blocked by MT3, the authors proposed that M_4 receptors could be responsible for the inhibition of dopamine-sensitive adenylyl cyclase in the striatum. However, we were not able to detect any significant effect on basal cyclic AMP production of either oxotremorine or carbachol in the striatum. It is evident that just a small fraction of adenylyl cyclase was stimulated by dopamine, both in the striatum and hippocampus, though (at 1 mM dopamine) that fraction was higher in the striatum (166 vs. 116% with respect to each basal level). If we consider the forskolin-stimulated adenylyl cyclase as total enzyme activity, we may conclude that a fraction of about 1.3% of the total cyclase activity would be regulated by dopamine in hippocampal membranes; and this fraction would be about 3.1% in striatal membranes. As a consequence, in the conditions of our experiments, it was not feasible to perform reliable assays to analyze the effect of selective agonists and antagonists in the hippocampus. Although it was rather difficult in the striatum, the results were more reliable than in the hippocampus. Oxotremorine inhibited by 49% the dopamine stimulated adenylyl cyclase activity in the striatum, respect to the basal value (see Table 1). That inhibition was fully reversed by MT3 (Fig. 4). Although the concentration of the toxin used was much higher than any receptor subtype selective concentration, MT3 has proved to have negligible or very low affinity for M_2 receptors [19], the subtype also proposed to be involved in adenylyl cyclase inhibition. Therefore some minor M_2 receptor contribution to the observed adenylyl cyclase inhibition could not be discarded.

On the other hand, we did not find any significant muscarinic effect on adenylyl cyclase activity in amygdala membranes, which could mean that the localization of the enzyme is different and/or there is not significant coupling with mAChR.

It has been reported by Sanchez-Lemus and Arias-Montaño that M_1 receptors would also be involved in regulation of cyclic AMP production through protein kinase C activation [39]. However, we did not find any cyclic AMP increase by either MT1 or MT2 addition. An explanation for this would be that those authors carried out their study in miniprisms while our membrane preparation did not preserve the biochemical and micro-architectural integrity, i.e., the corresponding substrates and effectors.

It was previously shown that MT1 and MT2—agonists at M_1 receptors—given into the rat hippocampus facilitated memory consolidation of an inhibitory avoidance task in the rat [18, 31]. MT3, highly selective for M_4, injected into the hippocampus was amnesic for the same task [19, 20]. Therefore, previous and present results—MT1 and MT3 antagonism at M_4—support the hypothesis that the M_4 receptor could be a positive modulator of memory.

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