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European Journal of Pharmacology 334 (1997) 43–47

ejp

Short communication

The muscarinic toxin 3 augments neuropeptide mRNA in rat striatum in vivo

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Received 12 June 1997; revised 15 July 1997; accepted 18 July 1997

Abstract

The selective M_4 muscarinic receptor toxin, MT3, was used in vivo to evaluate the role of M_4 receptors in cholinergic inhibition of neuropeptide mRNA expression in striatonigral neurons. Unilateral injection of the muscarinic toxin 3 (0.04–4 nmol) into the dorsal striatum of chronically-cannulated rats elevated basal levels of preprodynorphin, substance P and preproenkephalin mRNAs in the ipsilateral dorsal striatum as revealed by quantitative in situ hybridization. Pretreatment with muscarinic toxin 3 also augmented amphetamine (2.5 mg/kg, i.p.)-stimulated preprodynorphin and substance P expression in the dorsal striatum in a manner similar to that observed after the muscarinic antagonist, scopolamine. Since muscarinic toxin 3 has a much greater affinity for muscarinic M_4 receptors than for other subtypes, it is possible that muscarinic toxin 3, by interacting with the muscarinic M_4 subtype, regulates basal and/or dopamine-stimulated striatal neuropeptide gene expression. © 1997 Elsevier Science B.V.

Keywords: Dynorphin; Substance P; Enkephalin; Acetylcholine; Dopamine; Amphetamine; Nucleus accumbens; Caudoputamen; Behavior; Muscarinic receptors

1. Introduction

Progress has been made in investigating the regulation of striatal neuropeptide gene expression by cholinergic muscarinic receptors in recent years (Di Chiara et al., 1994; Wang and McGinty, 1996b). Recent data from this laboratory and others demonstrate that recent data from this laboratory and others demonstrate that pharmacological activation of muscarinic cholinoceptors suppresses constitutive and amphetamine-stimulated preprodynorphin and substance P mRNA expression in striatonigral neurons (Lucas and Harlan, 1995; Wang and McGinty, 1996a). In contrast, muscarinic receptor blockade induces basal, and augments amphetamine-stimulated, preprodynorphin and substance P gene expression (Wang and McGinty, 1996a). Thus, muscarinic receptors are negatively coupled to peptide gene expression in striatonigral neurons. Since the pharmacological agentonists and antagonists used in previous studies were non-selective in nature, it is unclear which subtype of muscarinic receptors is involved. Striatonigral neurons contain two subtypes of muscarinic receptors, M_1 and M_4 (Bernard et al., 1992; Weiner et al., 1990). The former stimulates phosphoinositol turnover and the latter inhibits cAMP formation (Hulme et al., 1990). Thus, the muscarinic M_4 subtype is the most likely receptor to inhibit striatonigral gene expression based on the positive linkage of the cAMP pathway to neuropeptide synthesis (Hyman et al., 1996). However, this speculation has not yet been confirmed because of the lack of muscarinic agents with satisfactory subtype selectivity.

Muscarinic toxin 3, a peptide toxin recently isolated from green mamba venom, has a high selectivity for cloned muscarinic m4 receptors (Jolkkonen et al., 1994). Compared to other known chemical compounds, such as himbacine, methoctramine and tropicamide, this toxin has a much greater ability to discriminate m4 from the other cloned muscarinic subtypes. In rat striatal membranes, the toxin has been confirmed to be a competitive antagonist of muscarinic M_4 receptors because it antagonizes the ability of acetylcholine to inhibit forskolin- and D_1 receptor-stimulated adenylyl cyclase activities because it antagonizes the ability of acetylcholine to inhibit forskolin- and

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dopamine D_1 receptor-stimulated adenylyl cyclase activities (Olianas et al., 1996). The weaker potency of muscarinic toxin 3 in antagonizing methacholine-stimulated [^{3}H]inositol phosphate formation in cerebral cortical membranes and acetylcholine-induced inhibition of adenyl cyclase in rat myocardium ''is consistent with the lower affinity of muscarinic toxin 3 for the cloned m1 and m2 receptor subtypes, respectively'' (Olianas et al., 1996).

In this study, muscarinic toxin 3 was microinjected into the rat striatum for the first time in vivo and its effects on basal and dopamine stimulation-induced preprodynorphin, substance P and preproenkephalin mRNA expression were evaluated by quantitative in situ hybridization in normal and amphetamine-stimulated rats, respectively.

2. Materials and methods

Adult male Wistar rats (225–249 g, Charles River, Raleigh, NC) were individually housed and maintained on a 12 h light/dark schedule with food and water provided ad libitum. All animal use procedures were in strict accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee.

Rats were anesthetized with chloral hydrate (400 mg/kg, i.p.) and chronically cannulated with a 24 gauge stainless steel guide cannula which penetrated to 2.5 mm above the injection site in the right striatum as described (Wang and McGinty, 1997). In the first experiment, six days after surgery, muscarinic toxin 3, which was purified by M.J. from the venom of *Dendroaspis angusticeps* (Jolkkonen et al., 1994) was infused ipsilaterally into the striatum in a dose range of 0.04–4 nmol/1 μl via a 30 gauge stainless steel cannula. In the second experiment, muscarinic toxin 3 (0.2 nmol/1 μl) or artificial cerebrospinal fluid (ACSF) was infused ipsilaterally into the striatum 20 min prior to i.p. injection of 2.5 mg/kg amphetamine (Sigma, St. Louis, MO) or saline. Muscarinic toxin 3 was dissolved in ACSF (in mM: NaCl 154, KCl 2.68, $MgCl_2$ 0.9 and $CaCl_2$ 1.22, pH 7.4).

In each experiment, 3 h after i.p. injection, when amphetamine-induced neuropeptide mRNA peaks (Wang and McGinty, 1995), the rats were anesthetized and decapitated. Quantitative in situ hybridization histochemistry was performed according to standard procedures in this laboratory (Wang and McGinty, 1996a). Quantitation of the mRNA hybridization signals on X-ray films was performed using NIH Image 1.44 (W. Rasband, NIMH). Briefly, the ^{14}C standards were measured, plotted against known dpm/mg, and converted to ^{35}S equivalents to generate a calibration curve. Film background was measured and corrected for uneven illumination. Under the density slice option, the hybridization signal in the entire dorsal striatum on the injected side was measured using a circle (diameter = 200 pixels). Quantitative changes were expressed as (1) the number of labeled pixels per area, (2) mean density of tissue in dpm/mg, and (3) integrated density which is the product of area times mean density.

A one-way analysis of variance (ANOVA) followed by a Bonferroni (Dunn) comparison of groups was performed on the area under curve (AUC) values calculated from plotting behavioral ratings against time. Significance in area, mean density, and integrated density between groups was determined by a nested two-way ANOVA followed by a Bonferroni (Dunn) comparison of groups.

3. Results

In experiment 1, muscarinic toxin 3 caused a dose-dependent increase in preprodynorphin, substance P, and preproenkephalin mRNA in the striatum in the lower dose range (0.04–0.2 nmol) which plateaued in the upper dose range (0.2–4 nmol) (Fig. 1A). In experiment 2, amphetamine elevated the hybridization signals of preprodynorphin (Fig. 2C versus Fig. 2A), substance P (Fig. 2G versus Fig. 2E) and preproenkephalin (data not shown) mRNAs in the dorsal striatum. The quantitative analysis of the 3 mRNA hybridization signals in the ipsilateral dorsal

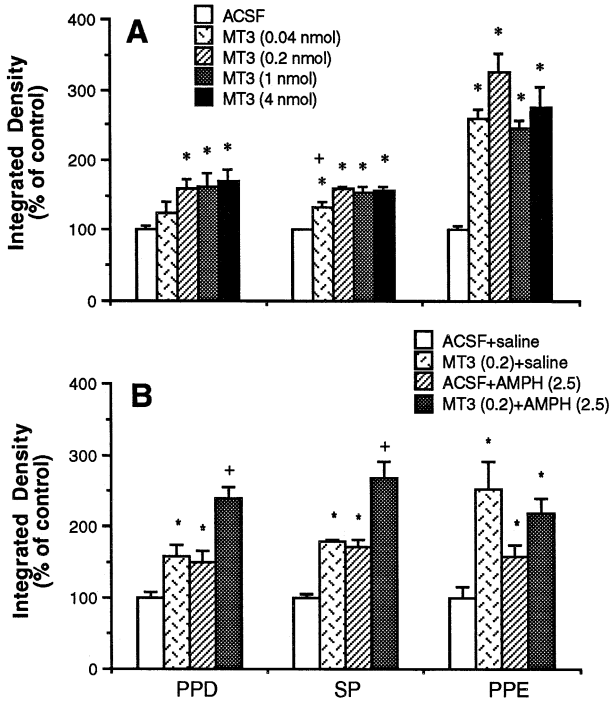

Fig. 1. (A) Quantitative analysis of the effects of intrastriatal muscarinic toxin 3 (MT3, 0.04–4 nmol) on constitutive expression of preprodynorphin (PPD), substance P (SP), and preproenkephalin (PPE) mRNAs in the dorsal striatum. (B) Quantitative analysis of the effects of intrastriatal MT3 (0.2 nmol) on amphetamine (AMPH, 2 mg/kg, i.p.)-stimulated PPD, SP and PPE mRNAs in the dorsal striatum. * $P < 0.05$ versus saline (A) or ACSF + saline (B), $^+$ $P < 0.05$ versus 0.2–4 nmol MT3 (A) or ACSF + AMPH (B).

striatum (Fig. 1B) confirmed that amphetamine increased preprodynorphin by 50%, substance P by 71%, and preproenkephalin by 58% above control values. Intrastriatal infusion of muscarinic toxin 3 prior to a systemic saline injection also elevated basal levels of preprodynorphin (Fig. 2B versus Fig. 2A), substance P (Fig. 2F versus Fig. 2E) and preproenkephalin (data not shown) mRNA expression in the injected dorsal striatum. The amplitude of muscarinic toxin 3-induced elevation was 58% for preprodynorphin, 79% for substance P, and 152% for preproenkephalin (Fig. 1B). When intrastriatal muscarinic toxin 3 was administered prior to systemic amphetamine, the expression of striatal preprodynorphin (Fig. 2D) and substance P (Fig. 2H) mRNA was augmented as compared to the expression of these mRNAs in ACSF + amphetamine-treated rats. The percent increases in preprodynorphin and substance P mRNA in the ipsilateral dorsal striatum after administration of muscarinic toxin 3 and amphetamine were 138% and 169%, respectively, which represented an additive increase as compared to that induced by either agent alone (Fig. 1B). For preproenkephalin mRNA, a 119% induction was seen after coadministration of muscarinic toxin 3 and amphetamine which did not significantly differ from that induced by either agent alone (Fig. 1B).

4. Discussion

Intrastriatal or systemic administration of the non-selective muscarinic receptor antagonist, scopolamine, increases basal and augments amphetamine-induced, striatonigral preprodynorphin/substance P peptide gene expression (Lucas and Harlan, 1995; Wang and McGinty, 1996a). In contrast, non-selective stimulation of muscarinic receptors with intrastriatal or systemic oxotremorine decreases stimulated preprodynorphin/substance P mRNA (Wang and McGinty, 1996a; Wang and McGinty, 1997). The opposite has been found for preproenkephalin-containing striatopallidal neurons, i.e., oxotremorine facilitates, and scopolamine, blocks, dopamine D_1 receptor-stimulated preproenkephalin mRNA. In contrast to scopolamine, mus-

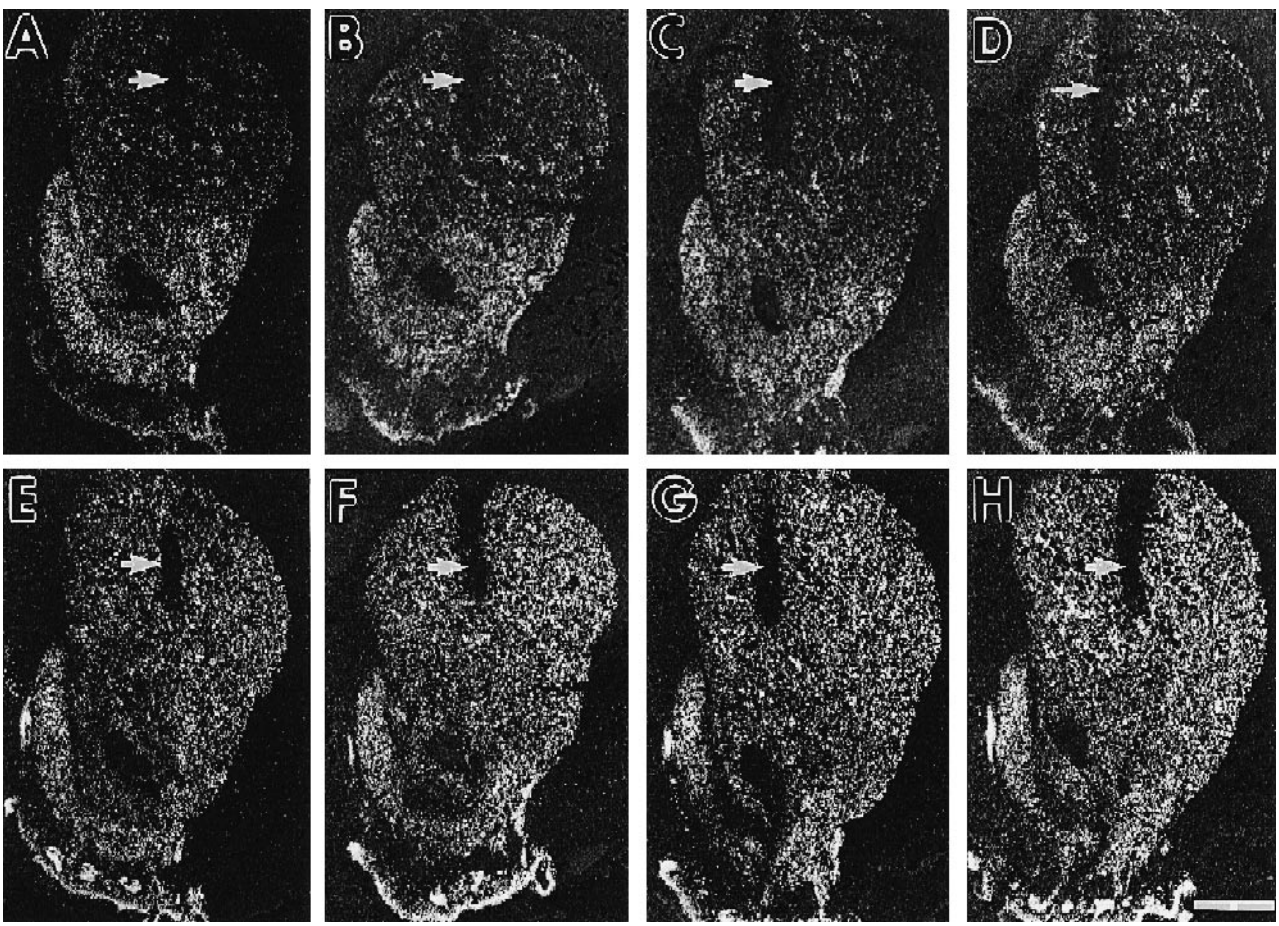

Fig. 2. Darkfield photomicrographs from emulsion-dipped slides illustrating the effects of intrastriatal muscarinic toxin 3 (0.2 nmol) on constitutive and amphetamine (2.5 mg/kg, i.p.)-stimulated preprodynorphin (A–D) and substance P (E–H) mRNA in the injected dorsal striatum. (A, E) ACSF + saline; (B, F) muscarinic toxin 3 + saline; (C, G) ACSF + amphetamine; (D, H) muscarinic toxin 3 + amphetamine. Arrows mark intrastriatal injection tracks. Scale bar, 1 mm.

carinic toxin 3 was found to increase basal levels of preproenkephalin mRNA in addition to preprodynorphin and substance P mRNA. The contrasting effects of muscarinic toxin 3 and scopolamine on basal preproenkephalin mRNA levels suggest that muscarinic toxin 3 discriminates between muscarinic receptors unlike scopolamine. Indeed, it is likely that muscarinic toxin 3 is exerting its effects on striatal gene expression selectively through muscarinic M_4 receptors because muscarinic toxin 3 has a higher affinity for M_4 than for other muscarinic receptor subtypes and M_4 is expressed by 100% of striatonigral and 39% of striatopallidal neurons. Because muscarinic M_4 receptors are negatively coupled to cAMP formation, their selective blockade should lead to an increase in gene expression in both types of medium spiny neurons by disinhibiting adenylyl cyclase.

An additional explanation resides in the presynaptic location of muscarinic M_4 receptors. M_2 and M_4 receptor immunoreactivity has been localized to striatal terminals which make asymmetric contacts and are presumably glutamatergic (Hersch and Levey, 1995). There is evidence that stimulation of muscarinic receptors decreases glutamatergic transmission (Hernandez et al., 1996) and glutamate efflux (Godhukin et al., 1984; Rawls and McGinty, 1997, in press) in the striatum. It is posssible, then, that muscarinic toxin 3 increases release of glutamate by removing muscarinic M_4 heteroceptor inhibition on striatal afferents. This action would favor glutamate receptor-stimulated augmentation of preproenkephalin, as well as preprodynorphin and substance P mRNAs, but, logically, scopolamine should have the same effect. An alternative explanation relies on the fact that striatal cholinergic interneurons contain muscarinic M_4 receptor mRNA (Bernard et al., 1992; Weiner et al., 1990) and muscarinic M_4 receptor immunoreactivity (Hersch and Levey, 1995) which may indicate that muscarinic M_4, along with muscarinic M_2, receptors function as presynaptic autoreceptors in the striatum. If muscarinic toxin 3 increases acetylcholine release in the striatum by blocking muscarinic M_4 receptors, muscarinic M_1 receptors on medium spiny neurons would be stimulated. In contrast, muscarinic M_1 receptors would not be available after non-selective blockade by scopolamine. Muscarinic M_1 receptors are coupled to stimulation of phosphoinositol turnover and are contained by almost all medium spiny neurons (Bernard et al., 1992; Weiner et al., 1990). Muscarinic M_1 receptor stimulation may favor induction of neuropeptide mRNA in both striatonigral and striatopallidal neurons. However, the in vivo selectivity of muscarinic toxin 3 cannot be confirmed until suitable muscarinic receptor subtype agonists become available against which to test muscarinic toxin 3. Therefore, the above explanations will remain speculative until further investigations are conducted to assess the precise contribution of muscarinic receptor subtypes and their pre- or post-synaptic localization to neuropeptide gene expression in medium spiny neurons.

5. Conclusion

Muscarinic toxin 3 (MT3), a peptide recently isolated from green mamba venom, has high selectivity for cloned m4 receptors. Recent studies have demonstrated that MT3 antagonizes forskolin- and D_1 receptor-stimulated adenylate cyclase activity. This study is the first attempt to utilize this toxin in vivo as a pharmacological antagonist for M_4 muscarinic receptors in which the contribution of M_4 receptors to the muscarinic inhibition of neuropeptide gene expression in striatal neurons was evaluated. It was found that pharmacological blockade of M_4 receptors by intrastriatal infusion of MT3 into the dorsal striatum increased constitutive PPD, PPE, and SP mRNA expression in the rat striatum, and augmented amphetamine-stimulated PPD and SP mRNA in striatonigral neurons. Thus, M_4 receptors exert an inhibitory influence on striatal peptide gene expression in both normal and/or dopamine-stimulated conditions.

Acknowledgements

This research was supported by DA03982. The authors wish to thank Denise C. Mayer and William T. Bohler for their technical assistance.

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