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The striatum and cerebral cortex express different muscarinic receptor mRNAs

Mark R. Brann, Noel J. Buckley[+] and Tom I. Bonner[+]

Metabolic Diseases Branch, National Institute of Diabetes, Digestive and Kidney Diseases, Building 10, Room 9c-101 and *[+] Laboratory of Cell Biology, National Institute of Mental Health, Building 36, Room 3A-17, National Insitutes of Health, Bethesda, MD 20892, USA*

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The existence of four distinct muscarinic acetylcholine receptor genes (m1 – m4) has recently been demonstrated. cDNAs for three of these receptors have been cloned from brain (m1, m3, m4) and one from heart (m2). To gain some understanding of the physiological role of the brain muscarinic receptors, we mapped the distribution of their mRNAs in rat brain by in situ hybridization. These mRNAs are barely detectable in the hindbrain and cerebellum. Within forebrain, each mRNA has a strikingly different pattern of distribution. The highest levels of m1 mRNA are in the cerebral cortex and hippocampus followed by the striatum. m3 mRNA is also prominent in the cerebral cortex, but has very low levels in the striatum. Conversely, the levels of m4 mRNA are highest in the striatum. Since the cognitive effects of muscarinic drugs have been localized to the cerebral cortex and hippocampus, and their psychomotor effects to the striatum, these data suggest that the muscarinic receptors which subserve these responses may be different gene products. Finally, we show that these muscarinic receptors can be distinguished pharmacologically, suggesting that it may be possible to develop drugs for the selective treatment of the psychomotor vs cognitive difficulties of Parkinson's and Alzheimer's disease, respectively.

Muscarinic receptor; Neurotransmitter receptor; Hybridization; mRNA; Neurological disease

1. INTRODUCTION

Due to their broad spectrum of action muscarinic drugs have been replaced by more selective agents in many clinical applications. For example, in part because of the peripheral and cognitive side-effects induced by antimuscarinic agents, they have been largely replaced by dopaminergic agents in the treatment of Parkinson's disease [1,2]. The discovery of heterogeneity of muscarinic receptors in binding and functional assays has encouraged intensive efforts for the development of more selective muscarinic agents. Unfortunately, with few exceptions, the classical approaches to drug development have met with little success, possibly due to the limited selectivity of the available drugs for putative subtypes, and the intimate mixture of multiple subtypes within tissues. The most notable exception is the antimuscarinic agent pirenzepine. Pirenzepine has high affinity for forebrain and low affinity for heart muscarinic receptors, these receptors having been designated M1 and M2, respectively [3]. The molecular cloning of two muscarinic receptor cDNAs, one from porcine brain [4] and the other from porcine heart [5,6], has confirmed a structural diversity associated with the M1-M2 classification, and has illustrated the utility of molecular genetics for the study of muscarinic receptor heterogeneity.

Based on the sequence of the brain cDNA we recently cloned three cDNAs from rat cerebral cortex (m1, m3, m4 [7]). Together with the cloned heart receptor (m2), these data illustrate the existence of four muscarinic receptors with different

Correspondence (present) address: M.R. Brann, Laboratory of Molecular Biology, National Institute of Neurological and Communicative Disorders and Stroke, Building 36, Room 3D-02, NIH, Bethesda, MD 20892, USA

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primary sequences. The human homologs of these receptors have also been cloned ([7] and unpublished). The m1, m3 and m4 receptors have high affinity for pirenzepine relative to the m2 receptors [6–8]. Thus, pharmacologically defined M1 muscarinic receptors consist of at least three gene products designated by lower-case letters (m1, m3, m4). To gain insight into the physiological roles of these receptors, we localized mRNA encoding them within rat brain. Data on the cardiac (m2) receptor are not presented here as we have cloned the human but not the rat homolog of the m2 receptor. However, using two 48 base cDNA probes, based on the human sequence, we have observed RNA with a size of ~6200 bases on Northern blots of RNA extracted from rat atria. Using these probes we have not detected any RNA on Northern blots of RNA extracted from several forebrain structures which were positive for m1, m3 and m4 RNA (in preparation).

2. MATERIALS AND METHODS

2.1. *Probes*

We prepared 45 or 48 base oligodeoxynucleotide probes complementary to sequences encoding amino acids 3–18 (m1), 2–17 (m3) and 3–17 (m4) of the rat muscarinic receptors [7]. The probes were made on an Appied Biosystems DNA synthesizer and purified by preparative gel electrophoresis. Each probe was labeled on the 3′-end with terminal deoxynucleotidyl transferase and deoxyadenosine triphosphate (labeled in the α position with ^{32}P for Northern blots, >3000 Ci/mmol, and ^{35}S for insitu hybridizations, >1000 Ci/mmol, New England Nuclear). Using a sequencing gel we have observed that the most common length of our oligonucleotide 3′-tails was 10 bases.

2.2. *In situ hybridizations*

In situ hybridizations were performed as described for localization of G-protein [9] and neuropeptide mRNAs [10]. Briefly, 12 μm sections of rat brain were prepared, mounted on gelatin-coated slides and stored at −70°C until use. Slides were warmed to room temperature, acetylated, dehydrated and extracted with chloroform. $(1–2)\times10^6$ dpm of labeled probe was added to each section in 50 μl hybridization buffer [4×SSC (1×SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.2), 50% formamide, 1×Denhardt's (0.02% Ficoll, 0.02% polyvinylpyrolidone, 0.02% bovine serum albumin), 250 μg/ml sheared single-stranded salmon sperm DNA, 100 mM dithiothreitol, and 10% dextran sulfate]. Slides were incubated overnight in a humid chamber at 37°C, and washed at 55°C with 1×SSC. The slides were dried and exposed to X-ray film for 5 weeks. The specificity of each probe was verified on Northern blots of total RNA performed under identical stringencies to those used for in situ hybridizations [9,10]. None of the probes labeled RNA extracted from heart, kidney or liver. In RNA extracted from cerebral cortex each probe labeled a single mRNA species of 3100, 4500 and 3300 bases for m1, m3 and m4, respectively, and their relative levels were m1>m3>m4.

2.3. *COS cell transfections*

COS-7 cells were transfected with 20 μg plasmid DNA by calcium phosphate precipitation [11]. Cells were plated 24 h before transfection (2×10^6 cells per 10 cm^2 plate), media were changed 24 h after transfection, and membranes harvested 48 h later. The plasmid DNA used for transfections consisted of the pcD expression vector which included the SV40 promoter. The DNA constructs which encode the muscarinic receptors have been described [7].

2.4. *Binding assays*

Binding assays were adapted from established procedures as previously described [12]. Assays were performed in 0.5 ml buffer [10 mm Hepes, 5 mM $MgCl_2$, (pH 7.4)] at room temperature with ~0.01 mg cos-7 cell membrane protein. Receptors were labeled with 100 pM [^{3}H]QNB and incubated with 10 separate concentrations of carbachol for 2 h. The affinities of [^{3}H]QNB for the three populations of binding sites are similar, and have been reported [7].

3. RESULTS

The distributions of m1, m3 and m4 mRNA in coronal (left) and sagittal (right) sections of rat brain are illustrated in fig.1. The concentrations of m1 and m3 mRNA were highest within the cerebral cortex (CX) and hippocampus (H), and within these brain regions they displayed distinct patterns of distribution. m1 mRNA was relatively evenly distributed throughout the cerebral cortex, while m3 mRNA was present in a more layered pattern. Central layers of the cerebral cortex have the lowest levels of m3 mRNA. Within the hippocampus, m1 mRNA was abundant within fields CA 1-3 of Ammon's horn and the dentate gyrus as illustrated in the sagittal sections of fig.1 (fields CA 1–3 circumscribe the dentate gyrus in the illustrated sections). m3 mRNA was also abundant within fields CA 1-3 of Ammon's horn, but was not detected within the dentate gyrus. m4 mRNA was present at low levels in the cerebral cortex and hippocampus and at high levels within the caudate putamen and nucleus accumbens (collectively referred to below as the striatum); and olfactory tubercle (ventral to the striatum in all illustrated sections). m1 mRNA was also present in the striatum, but at lower levels than those in the cerebral cortex and hippocampus. m3 mRNA levels were very low in the striatum relative to the

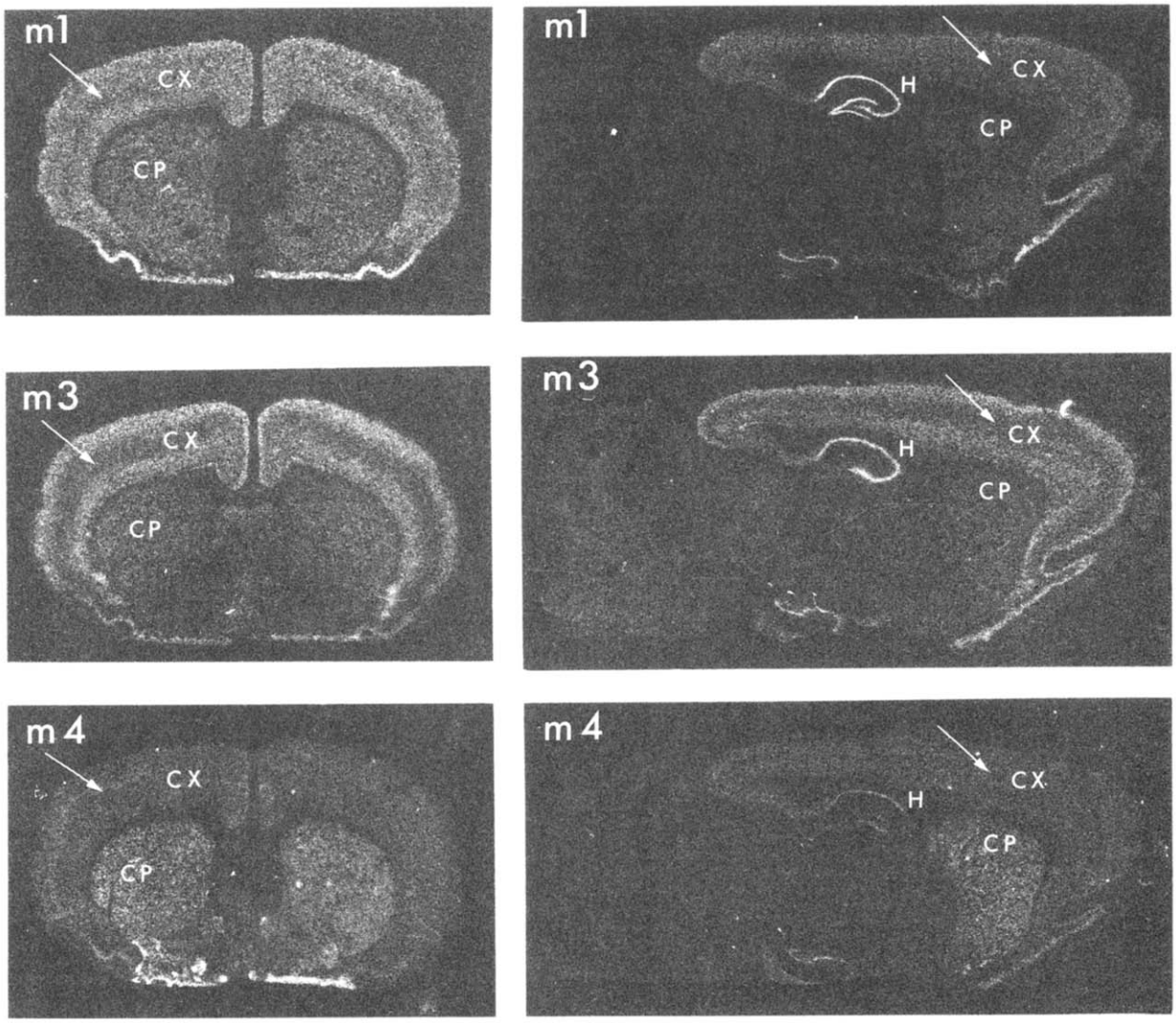

Fig.1. Distribution of muscarinic receptor mRNAs in rat brain. Serial 12 μm coronal (left) and sagittal (right) sections of rat brain were hybridized with synthetic ^{35}S-labeled oligodeoxynucleotide probes complementary to m1, m3 and m4 mRNA. Sections were washed at high stringency and exposed to X-ray film for 5 weeks. Regions rich in mRNA are light in the photomicrographs. Nonspecific hybridization was not detectable at this exposure time as defined with probes to mRNAs not expressed in brain (opsin and transducin). The illustrated sections correspond to coronal level interaural 10.00 mm and to sagital level lateral 2.90 mm as illustrated in the work of Paxinos and Watson [23]. Arrows indicate central layers of the cerebral cortex, which has little m3 mRNA. CX, cerebral cortex; CP, caudate putamen; H, hippocampus.

cerebral cortex and hippocampus. Overall, these data suggest that the majority of muscarinic receptors in the striatum are different gene products from those in the cerebral cortex and hippocampus.

Fig.2 illustrates the affinities of the muscarinic agonist carbachol for m1, m3 and m4 receptors. These receptors were transiently expressed in cos-7 cells and labeled with 100 pM [^{3}H]QNB. We have previously shown that these receptors have similar affinities for [^{3}H]QNB [7]. The affinity of carbachol for the m4 receptor is approx. 10-fold higher than for m1 and m3 receptors. The IC_{50} values are 201 μM (m1), 125 μM (m3) and 10.2 μM

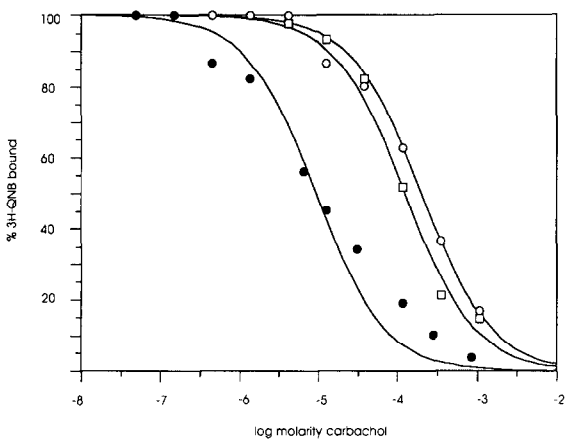

Fig.2. Binding of carbachol to muscarinic receptors expressed in COS-7 cells after transfection with muscarinic receptor cDNAs: m1 (○), m3 (□), m4 (●). Lines are computer-generated best fits of the data to the function % [^{3}H]QNB bound = $100-(100x/IC_{50})/(1+x/IC_{50})$, where x is carbachol concentration.

(m4). Overall, these data indicate that the muscarinic receptors encoded by mRNA which is most abundant in the striatum have higher affinity for carbachol than those which are most abundant in the cerebral cortex and hippocampus.

4. DISCUSSION

The composite of the distributions of m1, m3 and m4 mRNAs is in general agreement with previous autoradiographic examinations of muscarinic ligand binding to M1 muscarinic receptors in brain. M1 muscarinic receptors have been reported to be abundant in the hippocampus, cerebral cortex and striatum, but have low levels in the midbrain and hindbrain [13,14]. Detailed comparisons of receptor and mRNA distributions are complicated by several factors including the multiplicity of muscarinic receptors and lack of knowledge of the relationship of the levels of individual receptor mRNA to those of encoded protein. Also, unlike receptor proteins which are found in all parts of neuronal cells, mRNAs are present in cell bodies and proximal dendrites but not in distal dendritic fields and terminals which may project to different areas of the brain [10].

Muscarinic drugs exert distinct behavioral effects which are mediated by the striatum vs the cerebral cortex. For example, injection of muscarinic agonists in the striatum stimulates psychomotor behavior and potentiates the catalepsy induced by dopamine antagonists. Conversely, muscarinic antagonists block the catalepsy induced by dopamine antagonists [15,16]. In addition to playing a role in mediation of psychomotor behavior via sites within the striatum, these receptors are also associated with cognitive function. For example, muscarinic agonists improve memory and learning [17], while antagonists disrupt both these forms of behavior [18]. Also, lesions of cholinergic pathways to the cerebral cortex and hippocampus disrupt these measures of cognitive function and muscarinic agonists reverse these lesion-induced deficits, suggesting that muscarinic drugs alter cognitive function via sites within the cerebral cortex and hippocampus [17].

In humans, antimuscarinic agents improve the psychomotor performance of Parkinson's patients [1] but disrupt their cognitive ability [2]. On the other hand, decreases in cholinergic markers have been observed in the hippocampi and cerebral cortices of patients with Alzheimer's disease, leading to the proposal that cholinergic agonists may be useful in the treatment of these patients [19–21]. It is tempting to speculate that if drugs can be developed which are selective for the individual muscarinic receptor subtypes, they will be useful in treating these diseases. That is, an m4 antagonist may selectively improve psychomotor performance, and an m3 agonist may selectively improve cognitive function.

While the pharmacological characterization of the cloned muscarinic receptors is at a very early stage, it is clear that the receptors can be pharmacologically distinguished. In addition to carbachol, we have shown that the muscarinic antagonist pirenzepine discriminates among these receptors. That is, pirenzepine has approx. 4-fold lower affinity for m3 than for m1 and m4 receptors [7]. Pirenzepine and carbachol do not have the clinically desired selectivities, as an m3-selective agonist and an m4-selective antagonist would be the most promising candidates for the treatment of Alzheimer's and Parkinson's disease, respectively. The recent establishment of cell lines which stably express individual cloned muscarinic receptors should accelerate the development of drugs with these selectivities [6,12,22].

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