Localization of Muscarinic Receptor mRNAs in Rat Heart and Intrinsic Cardiac Ganglia by In Situ Hybridization

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Abstract Although the heart is considered a relatively pure source of m2 muscarinic receptors, the possible expression of other muscarinic receptor genes at discrete sites within the myocardium or by intrinsic cardiac ganglia had not been evaluated. Accordingly, the present study used in situ hybridization histochemistry with \(^{35}\)S-labeled oligonucleotide probes to address this issue. Initial experiments demonstrated that the localization of m2 mRNA was similar to that reported for muscarinic receptors labeled with the nonselective muscarinic antagonist quinuclidinyl benzilate; however, there were two important exceptions. The conducting system contained less message than expected, whereas the intrinsic cardiac ganglia contained more. The mismatch between muscarinic receptor and m2 mRNA densities in the conducting system could not be explained by the local expression of other muscarinic receptor genes, since m1, m3, and m4 mRNAs were not detected at this latter location. Accordingly, evaluation of other sites within the myocardium. However, the presence of a high density of prejunctional muscarinic receptors in the conducting system would be consistent with such a mismatch. Surprisingly, the intrinsic cardiac ganglia contained more than four times as much m2 mRNA as found in the atria. This level of message may be necessary for the production of prejunctional receptors on cholinergic nerve fibers within the heart and receptors localized to the ganglion cell bodies. The ganglia also contained smaller amounts of m1 and m4 mRNAs. These observations suggest that prejunctional muscarinic receptors could have a prominent role in regulating cholinergic neurotransmission in the conducting system and that multiple muscarinic receptors are present in the intrinsic cardiac ganglia. (Circ Res. 1994;75:813-820.)

Key Words • muscarinic receptor • mRNA • in situ hybridization • heart • cardiac ganglia

Muscarinic receptors located at a diversity of sites are known to contribute to the neuroregulation of cardiac function. Postjunctional cholinergic receptors on the myocardium have been most thoroughly studied and can directly elicit negative chronotropic, dromotropic, or inotropic responses when activated by muscarinic agonists or the stimulation of cholinergic nerves.1-3 Muscarinic receptors are also associated with cholinergic and adrenergic nerves in the heart.4-9 These prejunctional receptors have an inhibitory influence on the release of acetylcholine and norepinephrine and presumably have a role in fine-tuning the amount of neurotransmitter available to interact with postjunctional receptors. Last, muscarinic receptors are present on efferent neurons of the cardiac ganglia, where they may have a role in modulating ganglionic neurotransmission.10-12

An additional level of complexity has been added by the discovery of multiple muscarinic receptor subtypes. Five muscarinic receptor genes have been identified in molecular cloning experiments, and the corresponding receptor proteins have been designated m1 through m5.13,14 Heterogeneity of muscarinic receptors has also been documented in functional and radioligand binding experiments with muscarinic antagonists. Although the latter compounds exhibit only a modest degree of selectivity,15 they have permitted identification of four pharmacologic subtypes (ie, M1 through M4) of the muscarinic receptor.13,14,16 The M2 pharmacologic subtype of the muscarinic receptor is identical to the m2 molecular form,13,14 and various lines of evidence indicate that this is the dominant or only subtype present in the mammalian heart.13,14,17,18 However, the evaluation of muscarinic receptor mRNAs in the heart has been limited to the atrium, which is also the region of heart that has been most frequently used in functional and radioligand binding studies of muscarinic receptor subtypes.13,14 Accordingly, it is still possible that other muscarinic receptor genes might be expressed at discrete sites within the myocardium.

The distribution of muscarinic receptors in rat heart was evaluated in a previous study by in vitro receptor autoradiography with \(^{[3]}\)H]quinuclidinyl benzilate (QNB), a nonselective muscarinic antagonist.19 These experiments revealed a regional variation in the density of muscarinic receptors within the heart, with levels being highest in the atria, sinoatrial (SA) node, atrioventricular (AV) node, bundle of His, and intrinsic cardiac ganglia. The present study was initiated to evaluate the localization of m2 mRNA in rat heart by in situ hybridization histochemistry and to determine the extent to which the distribution and abundance of message for this receptor subtype correspond to that of muscarinic receptors. These experiments demonstrated that the conducting system contained less m2 mRNA than expected on the basis of its known high density of muscarinic receptors. Accordingly, additional experiments were done to determine whether other musca-
Muscarinic receptor genes are expressed at specific sites in the myocardium.

Materials and Methods

Twenty-two male Sprague-Dawley rats (275 to 510 g) were used in the present study according to a protocol approved by the University Committee on Animal Care. Animals were deeply anesthetized with sodium pentobarbital (75 mg/kg IP) before decapitation and removal of the heart and brain. The heart was washed briefly in cold sterile saline, and both organs were frozen on brass specimen plates using O.T.C. compound (Baxter Diagnostics) and dry ice. Frozen transverse sections of heart were collected, at intervals of ~100 μm, beginning at the anterior pole and continuing through the bundle of His. Adjacent 10- and 40-μm sections were used for in situ hybridization and acetylcholinesterase (AChE) histochemistry, respectively. Sections were thaw-mounted onto glass slides that were coated twice with chrome-alum gelatin containing 0.02% diethyl pyrocarbonate. Slides were transferred to boxes on dry ice immediately after sections were air-dried, and boxes were stored at ~70°C.

The localization of m2 mRNA was determined by the general procedure described by Young et al. A 48-base oligonucleotide probe (Table 1) with a sequence complementary to a region at the 3‘ end of rat m2 mRNA was purchased from DuPont NEN. This probe was 32P-labeled at the 3’ end by a tailing reaction with [α-32P]dATP and purified by using a label kit and a kit obtained from the same supplier. Tissue sections were fixed in 4% paraformaldehyde (5 minutes, pH 7.4), acetylated with 0.25% acetic anhydride (10 minutes), and dehydrated in a graded series of alcohols. Each slide was coated with ~200 μL of hybridization buffer containing 50% deionized formamide, 4× saline-sodium citrate buffer (SSC), 1× Denhardt’s solution, 100 mmol/L dithiothreitol, 10% dextran sulfate, 500 μg sonicated salmon sperm DNA per milliliter, 250 μg yeast tRNA per milliliter, and 32P-labeled probe at 6 to 22 million cpm/mL. The coated sections were covered with a strip of Parafilm and incubated for ~20 hours at 37°C in a humid chamber. After hybridization, the slides were dipped briefly in 1× SSC to remove the Parafilm, washed once for 1 hour in 0.5× SSC at 55°C and twice for 1 hour in 1× SSC at room temperature, rinsed with distilled water, dehydrated with 70% and 95% ethanol, and dried with a fan. Probes binding sites were initially identified by film autoradiography using Ultrofilm H (Mager Scientific) and exposure times of 3 to 7 weeks at 4°C. Exposure times were determined empirically and adjusted according to the activity of the probe. To obtain higher-resolution autoradiograms, the same slides were subsequently dipped into Kodak NTB-3 nuclear tract emulsion diluted 1:1 (vol/vol) with distilled water. These emulsions were developed after exposures that were five times longer than used for the films. Sections were stained with hematoxylin and eosin after the emulsions were fixed.

Four 10-μm sections of brain were routinely cut in either the parasagittal plane or in the transverse plane at the level of the anterior hippocampus. The localization of probe binding sites in these sections was compared with that reported by Buckley et al. This provided a positive control and a test for selectivity of the probe. Nonspecific binding of probe was routinely evaluated by pretreating representative sections of heart and brain with RNase A (8.6 U/mL, 45 to 60 minutes at 37°C, Sigma Chemical Co) to eliminate mRNA.

Adjacent 40-μm sections were stained for AChE according to the method of Koelle. Sections were preincubated with 1 μmol/L tetraosopropyl pyrophosphoramide (Sigma) for 30 minutes at 37°C to inhibit pseudocholinesterase. Incubations with acetylthiocholine substrate were for 3 hours at 37°C. The AChE stain was used to demonstrate cholinergic neurons of the intrinsic cardiac ganglia and regions of the myocardium that receive a dense cholinergic innervation (ie, the SA node and conducting systems). These slides were used for anatomic reference when evaluating autoradiograms.

Films were initially viewed under a microscope at magnifications of ×7 to ×30 for a qualitative evaluation of regional grain density and the effect of RNase pretreatment. A light box was used for illumination. The anatomic localization of grains in the films was determined by placing films over reference slides and aligning film images with the appropriate stained sections (ie, hematoxylin and eosin stain of same section that was used to generate image and AChE stain of adjacent section). Camera lucida drawings were also made to help identify small regions. A microcomputer-assisted imaging device (Imaging Research Inc) was subsequently used to quantify grain density associated with specific sites. Images on the films were displayed on a video screen and automatically digitized into 256 arbitrary gray levels. Since gray levels vary inversely with grain density, readings were automatically converted to relative optical density (ROD) according to the following equation: ROD= log10 (256 gray levels/sample gray levels). Regions of interest were outlined by a square with 100-μm sides (heart) or a circle of approximately the same diameter (ganglia), and the corresponding ROD was recorded. For each area of interest, multiple measurements were obtained from untreated and RNase-treated sections. Readings were also obtained from adjacent regions of film to determine background. After subtracting background, ROD values from untreated sections reflected the total number of m2 probe binding sites for a region; RODs from sections treated with RNase were due to nonspecific binding of the probe. Specific binding of the m2 probe was determined by subtracting nonspecific binding from the total. Since nonspecific binding did not vary significantly between regions within experiments, the average value for all regions was used to calculate specific binding when a matched value for that region was not available.
Since the specific activity of probes and exposure times varied between experiments and radioactive standards were not used in the study, regional specific binding values were normalized to the average value for right and left atrium in each experiment. The general linear models procedure for two-way ANOVA of unbalanced data was used for initial evaluation of the normalized data, since some regions were not present in sections from all animals (PC version of SAS, SAS Institute Inc). Duncan's multiple range test was used for subsequent pairwise comparisons. The criterion for statistical significance was \( P < .05 \).

Additional in situ hybridization experiments were done to evaluate the possibility that other muscarinic receptor genes might be expressed at discrete sites in the myocardium. Probes for m1, m3, and m4 muscarinic receptor mRNAs (Table 1) were purchased from DuPont NEN and used as described previously with detection by film autoradiography. At least three animals were used to evaluate the binding of each probe to sections of heart and brain.

**Results**

Regional variation in the density of m2 mRNA probe binding to heart and brain was evident on visual examination of film autoradiograms (Figs 1 and 2A) and microscopic evaluation of nuclear emulsion autoradiograms (Fig 3). Pretreatment with RNase eliminated specific binding of probe to mRNA and resulted in homogeneous patterns of grain distribution due to nonspecific binding sites in the tissue sections (Figs 1A and 1B and 2A and 2B). The density of m2 mRNA was clearly higher in the atrial myocardium than in the ventricular myocardium (Fig 1A and 1D). However, within each of these tissues, the abundance of binding sites for the m2 probe appeared to be relatively uniform. Quantitative analysis of regional grain densities in film autoradiograms confirmed these impressions, with the cardiac regions falling into two distinct groups according to normalized specific m2 probe binding (Table 2). The average density of m2 mRNA (ie, normalized specific probe binding) in right and left atrium was approximately three times greater than values measured at various locations within the ventricular myocardium. Comparison of grain densities in the right atrium, left atrium, and SA node revealed no significant differences. However, as noted in a previous study,19 cholinergic nerve fibers were more abundant in the right atrium than in the left and were particularly dense in the region of the SA node. Among atrial regions, the average density of m2 mRNA was lowest in the interatrial septum, but this difference did not achieve statistical significance. No differences in m2 mRNA abundance were detected when values were compared for right and left ventricles, interventricular septum, bundle of His, and the AV node. In marked contrast, the highest density of AChE-stained nerve fibers in rat heart was found in the AV node and bundle of His (Fig 1C).

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**Fig 1.** Photographs showing the localization of m2 mRNA and acetylcholinesterase (AChE) in transverse sections of rat heart. Panels A, B, and D were made by using film autoradiograms as negatives, so silver grains appear white on a black background. A, m2 probe binding to section containing atrial and ventricular myocardium and intrinsic cardiac ganglia. B, m2 probe binding to adjacent section pretreated with RNase. C, AChE-stained section from another heart showing dense cholinergic innervation to the atrioventricular node (av) and bundle of His (arrow). D, Localization of m2 probe binding sites in section adjacent to C. la indicates left atrium; ra, right atrium; rv, right ventricle; and arrowhead, ganglion. Bars=2 mm.
Labeling of cardiac myocytes was evident in nuclear emulsion autoradiograms (Fig 3A and 3B).

Intrinsic cardiac ganglia were present in several of the sections through the heart and were highly labeled by the m2 mRNA probe (Fig 1A). Image analysis of film autoradiograms established that the grain density over these ganglia was more than four times greater than that found in the atria (Table 2). Silver grains were highly concentrated over ganglion cell bodies in the nuclear emulsion autoradiograms (Fig 3C).

Specific binding of the m2 mRNA probe was identified in the pontine nucleus and motor nuclei of the fifth and seventh cranial nerves (Fig 2A and 2B) but was not detected in forebrain regions such as dorsal hippocampus and neocortex (not shown). These observations are in agreement with a previous report by Buckley et al.11 on the distribution of m2 mRNA in rat brain and confirm the specificity of this probe.

The conducting system and other regions of myocardium did not contain specific binding sites for m1, m3, or m4 mRNA probes (Fig 4), whereas such sites were evident in brain sections processed along with the hearts (Fig 2C through 2E). Furthermore, the localization of probe binding sites in the brain was identical to that previously reported by other investigators.21 Specific binding sites for m1 and m4 mRNA probes were also detected in the intrinsic cardiac ganglia, but they were far less abundant than sites for the m2 mRNA probe (compare Fig 4A and 4B and Fig 1A).

Specific labeling of cells in the coronary vasculature was not detected with the m2 mRNA probe. Likewise, specific labeling of blood vessels by m1, m3, or m4 probes could not be distinguished in film autoradiograms.

**Discussion**

The present study has used a specific oligonucleotide probe to demonstrate the regional and cellular localization of mRNA encoding the m2 muscarinic receptor in rat heart. Many of the results are consistent with our previous autoradiographic findings for regional density of muscarinic receptors labeled with [3H]QNB.19 In particular, m2 mRNA was more abundant in the atria than in the ventricles but uniform in density throughout these regions. However, there was a prominent mismatch of m2 mRNA and muscarinic receptor densities at two sites, the conducting system and the intrinsic cardiac ganglia. The conducting system (ie, AV node and bundle of His) contained less m2 mRNA than predicted on the basis of the density of muscarinic receptors, whereas the intrinsic cardiac ganglia contained more than expected.

The AV node and bundle of His could not be distinguished from the adjacent ventricular myocardium on the basis of grain densities in autoradiograms for m2 mRNA in the present study. These regions were clearly visible in autoradiograms showing [3H]QNB binding sites.19 Therefore, compared with other regions of the heart, the conducting system does not appear to have
enough m2 message to account for the total amount of muscarinic receptors present. All muscarinic receptor subtypes present in a tissue are labeled with [3H]QNB, since it is a nonselective muscarinic antagonist, but the oligonucleotide probe labeled only one of five mRNAs. Consequently, one possible explanation for the discrepancy between mRNA and receptor concentrations was that additional muscarinic receptor genes might be expressed by myocytes of the AV node and bundle of His. The most likely candidates were the m1, m3, and m4 genes, since they are commonly expressed, but the corresponding mRNAs were not detected at any myocardial site in additional in situ hybridization experiments with selective oligonucleotide probes. These probes were clearly able to identify their targets, since the appropriate mRNAs were detected in control sections of brain. Preliminary in situ hybridization experiments with an oligonucleotide probe directed at m5 mRNA have been unsuccessful, but expression of the m5 muscarinic receptor gene is extremely rare. Therefore, the myocytes of the conducting system probably contain only m2 muscarinic receptors.

A more plausible explanation for the relatively low density of m2 mRNA in the conducting system is that many of the muscarinic receptors identified by autoradiography may have a prejunctional localization on cholinergic and/or adrenergic varicosities. It is well known that the conducting system is densely innervated by both adrenergic and cholinergic nerves. Although neurons are known to have receptors localized to the plasma membrane of the cell body and peripheral processes, their mRNA is restricted to the cytoplasm of the cell body and proximal dendrites. Consequently, a mismatch of receptor and mRNA densities would be expected if a large fraction of the muscarinic receptors in the conducting system are prejunctional.

Lack of correspondence between levels of receptor and message might also be attributed to differential regulation of their synthesis and/or degradation. However, available evidence suggests the opposite for muscarinic receptors. There is generally a good correlation between the presence and abundance of muscarinic receptor mRNA and the corresponding protein in peripheral tissues and the central nervous system. Furthermore, studies evaluating muscarinic receptor regulation in cultured chick heart cells have demonstrated that levels of muscarinic receptor protein and the corresponding mRNA change in parallel when the cells are exposed to agonist. In this model system, muscarinic receptor activation can cause an increase in receptor degradation and a decrease in the transcription rate of muscarinic receptor genes. Accordingly, there appears to be some linkage in the regulation of message and protein levels for muscarinic receptors.

Prejunctional receptors are presumably present on cholinergic nerve fibers throughout the heart, but we
could not detect an obvious mismatch between densities of receptor and message at other sites such as the SA node. Regional differences in the density of cholinergic innervation and abundance of myocardial muscarinic receptors are two factors that could contribute to this circumstance. Although cholinergic nerve fibers are more plentiful in the SA node than in other regions of atrium, the conducting system has a much higher density of these nerves compared with all other areas of rat heart. If the density of postjunctional muscarinic receptors did not vary between regions of the heart, then prejunctional receptors would be expected to constitute a greater percentage of the total population in the conducting system compared with the SA node solely on the basis of its greater density of innervation. However, atrial myocytes may contain more muscarinic receptors than myocytes in the conducting system and ventricles. A higher density of postjunctional receptors would further reduce the percentage of the total receptor population contributed by prejunctional receptors and decrease our ability to detect a disproportionality between the levels of receptor and message.

The subtype of muscarinic receptor present on adrenergic and cholinergic nerve fibers in the heart has been evaluated by pharmacologic methods in experiments with the isolated rat heart preparation. Bognar et al. found that the amount of acetylcholine released in response to vagal stimulation was increased in the presence of the M3 antagonists, AF-DX 116 and methoctramine, but not in the presence of hexahydrasiladifenidol, an M2 antagonist. This pattern of sensitivity suggests that autoinhibition of acetylcholine release in the heart is mediated by M1 receptors. The results are less clear in regard to the prejunctional muscarinic receptors on adrenergic nerves, since M1, M2, and M3 antagonists were examined in different studies and all had some ability to block the effect of muscarinic agonists to inhibit norepinephrine release. Nevertheless, AF-DX 116 had the highest pA2 among the selective antagonists studied, suggesting that prejunctional muscarinic receptors on cardiac adrenergic nerves may also be of the M1 subtype.

The highest density of M2 mRNA in the heart was found in the intrinsic cardiac ganglia. Specific probe binding to this region was more than four times higher than values recorded for atrial myocardium and was concentrated over cell bodies of the postganglionic neurons. The former point was initially surprising, since
we previously found equal numbers of [3H]QNB binding sites (ie, muscarinic receptors) in the atria and ganglia. However, neurons in the intrinsic cardiac ganglia need to provide muscarinic receptors for a greater area of cell membrane compared with cardiac myocytes. Receptors destined for transport to the somatic, dendritic, and prejunctional membranes would all be made within the cell body and proximal dendrites. The greater synthetic demand that this would impose compared with that in myocytes would presumably account for the higher m2 mRNA levels.

We also identified m1 and m4 mRNAs in the cardiac ganglia, but they were less abundant than m2 mRNA. No binding of the m3 probe to the cardiac ganglia was observed in the present study. This differential labeling of the cardiac ganglia by four distinct oligonucleotides of similar size constitutes additional evidence that the high amount of m2 probe binding at this site truly reflects the abundance of m2 mRNA and cannot be attributed to nonspecific binding of oligonucleotides to neuronal mRNA.

The presence of multiple muscarinic receptor mRNAs in the intrinsic cardiac ganglia was also reported recently by other investigators. They were able to detect expression of m1, m2, m3, and m4 muscarinic receptor genes by intrinsic neurons of neonatal guinea pig and adult rat heart. Our results for cardiac ganglia are consistent with this report, but we were unable to detect m3 gene expression. The m3 probe used in the present study labeled the appropriate target mRNA at discrete sites in rat forebrain as effectively as our other probes labeled their targets. However, it is possible that the specific activity of our probe was insufficient to detect the amount of m3 mRNA present in the ganglia. This explanation is consistent with their suggestion that m3 and m4 mRNAs may be less abundant than message for other muscarinic receptors in the intracardiac neurons.

Stimulation of muscarinic receptors in cultured guinea pig intracardiac neurons causes a biphasic change in the membrane potential. The initial phase is a hyperpolarization that has been attributed to stimulation of M2 receptors, since it can be blocked by AF-DX 116 but is unaffected by pirenzepine. This response is followed by a depolarization mediated by M1 receptors. Therefore, different muscarinic receptor subtypes probably make unique contributions to the response of intracardiac neurons to cholinergic stimulation.

The abundance of m2 muscarinic receptors in the rat heart was recently evaluated in biochemical experiments that used selective antisera. That study demonstrated that 92% of the muscarinic receptors in a membrane preparation from homogenates of heart could be immunoprecipitated with m2 selective antiserum, indicating that they were of the m2 subtype. However, the authors regarded this as an underestimate and proposed that 100% of the receptors in heart may be of the m2 subtype. This conclusion is consistent with findings in the present study and the fact that only m2 mRNA has been detected in Northern analysis of rat atrium. Nevertheless, it is still possible that additional muscarinic receptor subtypes may be present in relatively low abundance on cardiac nerve fibers, since other muscarinic receptor mRNAs have been detected in the intrinsic cardiac ganglia. Additional experiments will be needed to resolve this issue.

In conclusion, the regional density of m2 muscarinic receptor mRNA in rat heart shows many similarities to the pattern previously reported for the total muscarinic receptor population. However, values for the conducting system and intrinsic cardiac ganglia deviated from this pattern. The observation of relatively low levels of m2 mRNA in the conducting system may reflect the presence of a high density of prejunctional muscarinic receptors at this site, since expression of m1, m3, and m4 genes was not detected in heart. If this is true, then prejunctional muscarinic receptors could have a major role in regulating the amounts of neurotransmitter available to interact with postjunctional receptors in the conducting system. In contrast, the presence of relatively high levels of m2 mRNA in neurons within the intrinsic cardiac ganglia could result from the need to synthesize receptors for a large area of membrane, including both the cell body and peripheral processes. Smaller quantities of m1 and m4 muscarinic receptor mRNA were also detected in the cardiac ganglia, suggesting the possibility that multiple receptor subtypes are present at sites on the postganglionic neurons.

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