

ACCELERATED COMMUNICATION

Quantitation of mRNAs for M₁ to M₅ Subtypes of Muscarinic Receptors in Rat Heart and Brain Cortex

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ABSTRACT

It has been generally accepted that, of the five subtypes of muscarinic receptors (M₁–M₅), only the M₂ subtype is expressed in mammalian heart. This notion has recently been challenged by a series of reports indicating that mRNAs for some or all non-M₂ subtypes are also present in mammalian heart, in parallel with the M₂ mRNA. However, the quantities of relevant mRNAs reported to be present in the heart are not known, which makes it difficult to evaluate their likely significance. We measured the concentrations of the five muscarinic mRNAs by competitive reverse transcription-polymerase chain reaction and discovered that the M₂ mRNA represents more than 90% of total muscarinic mRNAs in rat atria and in either

ventricle. The concentrations of total muscarinic mRNAs and of the M₂ mRNA were more than twice as high in the atria than in the ventricles. mRNAs for all non-M₂ muscarinic receptor subtypes were also detected but represented less than 1% (M₁ and M₄), less than 3% (M₃), and less than 5% (M₅) of total muscarinic RNAs in the atria and ventricles. The findings support the concept of the prevalent role of the M₂ muscarinic receptors in the cholinergic control of the heart. When the same method of quantitation was applied to rat cerebral cortex, mRNAs for individual subtypes were found to represent 36% (M₁), 21% (M₂), 25% (M₃), 11% (M₄), and 7% (M₅) of total muscarinic mRNAs.

It seemed firmly established in the early 1990s that the population of muscarinic receptors in the mammalian heart is homogeneous and corresponds fully to the M₂ subtype (Caulfield, 1993). This view was mainly formed from an analysis of cDNA for cardiac muscarinic receptors (Kubo et al., 1986; Peralta et al., 1987b), Northern blots of cardiac mRNAs (Peralta et al., 1987a; Maeda et al., 1988; Pinkas-Kramarski et al., 1989), radioligand binding assays with steep competition binding curves (Doods et al., 1987; Giraldo et al., 1988; Deighton et al., 1990), and immunoassays (Dörje et al., 1991).

Gallo et al. (1993) were the first to report the presence of a non-M₂ mRNA in mammalian heart (namely, M₁) and were soon followed by others. In recent years, a series of reports (Table 1) has appeared that, taken together, challenge the established concept of the predominant role of the M₂ receptor subtype in the parasympathetic control of the heart in mammals. The possibility that the non-M₂ muscarinic receptors play more than a marginal role in the cholinergic control

of the heart attracts considerable attention, particularly because the involvement of such receptors might help explain some controversial features of cardiac cholinergic pharmacology (Brodde and Michel, 1999) and contribute to the understanding of the roles of different types of G proteins in the heart (Dorn and Brown, 1999). It can be seen in Table 1, however, that there are enormous variations in published data. Although some variations might be a result of differences between species, a regular pattern of the presence/absence of individual non-M₂ mRNAs is difficult to establish. The main difficulty in evaluating the significance of data on non-M₂ mRNAs in mammalian hearts stems from the fact that, up to now, they have never been quantitated. It is therefore difficult to distinguish between the presence of trace and perhaps incidental versus substantial amounts of the messenger.

The availability of quantitative data on the expression of individual muscarinic receptor subtypes seems crucial for evaluating and testing hypotheses about their functional roles in the heart and for studying mechanisms by which the expression is controlled. This stimulated us to investigate the concentrations of mRNAs for muscarinic M₁ to M₅ receptors

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in cardiac atria and ventricles of the rat using competitive RT-PCR. In parallel with the work on the heart (originally just as a type of control experiment), we also determined the concentrations of mRNAs for muscarinic M_1 to M_5 receptors in rat cerebral cortex.

Materials and Methods

Outline. The quantitation of mRNAs was performed by competitive RT-PCR (Piatak et al., 1996). The same primers were used to amplify simultaneously the cDNA corresponding to a selected segment of the native mRNA (the concentration of which had to be determined) and the cDNA corresponding to a competitor RNA (which had been prepared in advance and added to the RT mixture at several known concentrations). The competitor RNAs had base sequences identical to the segments of native M_1 to M_5 mRNAs used for RT-PCR but contained a 10 to 15% deletion near their 3' end to distinguish RT-PCR products of the native and the competitor RNA on agarose gels. Comparing the quantities of the amplification products of the native versus the competitor RNA permitted the computation of the number of molecules of the native RNA that had been present in the reaction tube at the start of RT-PCR.

Extraction of Total RNA. Adult male Wistar rats weighing 180 to 200 g were killed by decapitation, and their hearts were taken out rapidly. Cardiac ventricles were cut open, and the hearts were submerged into RNAlater solution (Ambion, Austin, TX) to prevent RNA degradation. After 10 to 20 min, the atria and the walls of the right and the left ventricles were separated (taking care to remove non-cardiac tissues adhering to the atria) and homogenized in glass/glass homogenizers in the RNA isolation reagent RNAwiz (Ambion), and then RNA was isolated according to the manufacturer's instructions. The precipitated RNA was resuspended in water, heated at 60°C for 10 min, treated with DNase I using the DNA-free reagent (Ambion), and stored at -70°C. The quality of RNA was assessed by denaturing agarose electrophoresis.

Primers for RT-PCR of Native M_1 to M_5 mRNAs. Single sets of primers were used in work with the M_2 to M_5 subtypes, but two different sets of primers (M_1A and M_1B) were tested in experiments with the M_1 subtype. Primer sequences were from known sequences of genes for rat M_1 (GenBank M16406), M_2 (J03025), M_3 (M16407), M_4 (M16409), and M_5 (M22926) muscarinic receptor subtypes (Bonner et al., 1987; Gocayne et al., 1987; Liao et al., 1989), and primers were synthesized by VBC-Genomics (Vienna, Austria).

Primer sequences were as follows (Wei et al., 1994; Sharma et al., 1996): M_1A -sense, 5'-ATG GCC GCC TTC TAC CTC CCT GTC-3'; M_1A -antisense, 5'-CGG TCT CGG CCT TTC TTG GTG G-3'; amplified segment, 373 bp. M_1B -sense, 5'-GCA CAG GCA CCC ACC AAG CAG-3'; M_1B -antisense, 5'-AGA GCA GCA GCA GGC GGA ACG-3'; amplified segment, 428 bp. M_2 -sense, 5'-TAC CCT CTA CAC TGT GAT TGG C-3'; M_2 -antisense, 5'-ATG ATG ACA GGC AGA TAG-3'; amplified segment, 363 bp. M_3 -sense, 5'-GTG GTG TGA TTG GTC TG-3'; M_3 -antisense, 5'-TCT GCC GAG GAG TTG GTG TC-3'; am-

plified segment, 790 bp. M_4 -sense, 5'-AGT GCT TCA TCC AGT TCT TGT CCA-3'; M_4 -antisense, 5'-CAC ATT CAT TGC CTG TCT GCT TTG-3'; amplified segment, 510 bp. M_5 -sense, 5'-CTC ATC ATT GGC ATC TTC TTC-3'; M_5 -antisense, 5'-GGT CCT TGG TTC GCT TCT CTG-3'; amplified segment, 451 bp.

Primers for the Production of DNA Templates Used for the Synthesis of Competitor RNAs. The sense primers were the same as those described in the preceding section except that the T7 promoter (5'-TAA TAC GAC TCA CTA TAG G-3') had been attached to their 5' ends.

The antisense primers included the whole sequence of corresponding antisense primers described in the preceding section at their 5' ends but were extended at their 3' ends to make a 10 to 15% deletion in the amplified segment compared with the segment from the native mRNA. Their sequences were as follows: M_1A competitor antisense, 5'-CGG TCT CGG CCT TTC TTG GTG GTG TGC TTC AGA ATC TAC C-3'; M_1B competitor antisense, 5'-AGA GCA GCA GCA GGC GGA ACG TGT TGA CGT AGC ATA GC-3'; M_2 competitor antisense, 5'-ATG ATG ACA GGC AGA TAG AGC ATT CCC CAT CCT CCA CAG-3'; M_3 competitor antisense, 5'-TCT GCC GAG GAG TTG GTG TCA CCC ATG CTC TTC TG-3'; M_4 competitor antisense, 5'-CAC ATT CAT TGC TCT GCT TTG AGC GCT GGA GTG GTG GCC-3'; and M_5 competitor antisense, 5'-GGT CCT TGG TTC GCT TCT CTG GGC AGC AAT GGC AGT CC-3'.

The final length of the competitors produced with these primers in the way described below was 323, 378, 308, 715, 442, and 397 bp in the case of the M_1A , M_1B , M_2 , M_3 , M_4 , and M_5 competitor antisense primers, respectively.

Production of Competitor RNAs. DNA templates for the synthesis of RNA competitors were generated in an RT-PCR reaction using native mRNA and the primers described in the preceding section. PCR products were cloned into the pCRII-TOPO vector (Invitrogen, Carlsbad, CA) or, in experiments with the M_1B primers, the pUC18 vector (Stratagene, La Jolla, CA). Plasmids were cut after their 3' ends and were used for in vitro transcription with T7 RNA polymerase (Roche Applied Science, Indianapolis, IN). After 2 h of incubation, samples were treated with DNA-free reagent (Ambion) to digest plasmid DNA with DNase I and also to inactivate and remove the enzyme. RNA was purified by polyacrylamide/8 M urea denaturing gel electrophoresis. The band representing a full-length transcript was excised; RNA was eluted and precipitated, resuspended in water, and its concentration was determined spectrophotometrically. The competitors were dissolved in carrier yeast tRNA (200 ng/ μ l), divided into aliquots, and stored at -70°C. Each aliquot was thawed only once. For quantitation experiments, competitor RNA was further diluted by carrier yeast tRNA (20 ng/ μ l).

Quantitation Experiments. RT reaction and PCR were performed separately. Reverse transcription occurred in a total volume of 10 μ l, comprising 1 μ g of total cardiac RNA, the RNA competitor (five or six different amounts were used for competitions), 1.25 μ M antisense primer, deoxynucleoside-5'-triphosphates at a concentration of 0.8 mM each, 5 mM dithiothreitol, 5% dimethyl sulfoxide, and

TABLE 1

References on the occurrence of mRNAs for multiple muscarinic receptor subtypes in mammalian heart

| Preparation and Method | M_1 | M_2 | M_3 | M_4 | M_5 | Reference |
|--------------------------------------------------|-------|-------|-------|--------|-------|--------------------------|
| Guinea-pig ventricles, RT-PCR | Yes | N.D. | No | No | N.D. | Gallo et al. (1993) |
| Guinea-pig atrial myocytes in culture, ISH | No | Yes | No | No | No | Hassall et al. (1993) |
| Intrinsic ganglia in rat heart, ISH | Yes | Yes | Yes | Yes | No | Hassall et al. (1993) |
| Rat heart muscle, ISH | No | Yes | No | No | N.D. | Hoover et al. (1994) |
| Intrinsic ganglia in rat heart, ISH | Yes | Yes | No | Yes | N.D. | Hoover et al. (1994) |
| Rat dispersed ventricular myocytes, RT-PCR | Yes | Yes | No | No | No | Sharma et al. (1996) |
| Rat single cultured ventricular myocytes, RT-PCR | Yes | Yes | No | Yes/No | No | Colecraft et al. (1998) |
| Canine atria, RT-PCR | No | Yes | Yes | Yes | N.D. | Shi et al. (1999) |
| Human atria and ventricles, RT-PCR | No | Yes | Yes | No | No | Hellgren et al., 2000 |
| Human atria and ventricles, RT-PCR | Yes | Yes | Yes | Yes | Yes | Wang et al. (2001) |
| Human atria, RT-PCR | No | Yes | Yes | No | No | Oberhauser et al. (2001) |

N.D., not determined; ISH, in situ hybridization.

3 units of C. therm. polymerase (Roche) with 2 μ l of the corresponding RT buffer. The incubation lasted 30 min at 60°C, was terminated by heating 3 min at 94°C, and tubes were then chilled on ice.

PCR occurred in a total volume of 50 μ l, comprising 5 μ l of the reaction mixture from reverse transcription, 0.25 μ M sense primer, 0.15 μ M antisense primer, 2.2 mM MgCl₂, deoxynucleoside-5'-triphosphates at a concentration of 150 μ M each, and 1.25 units Hot Start Taq polymerase (QIAGEN, Valencia, CA) with 5 μ l of the corresponding PCR buffer. The temperature profile was 15 min at 95°C, 35 s at 60°C, and 45 s at 72°C, followed by cycles of 45 s at 95°C, 35 s at 62°C, and 40 s at 72°C, and finished with 7 min at 72°C. The number of cycles was 28 for M₂, 35 for M₁ in the atria, 40 for M₁ in the ventricles, 35 for M₃ and M₅, and 38 for M₄.

Every experiment included two negative controls: (1) reverse transcription without RNA followed by PCR to check for contamination of reagents used in both steps; and (2) PCR with RNA but without reverse transcription to check for contamination with residual genomic DNA in RNA samples.

Part of the PCR mixture (usually 10 μ l) was subjected to electrophoresis in 2% agarose gel containing ethidiumbromide (0.2 μ g/ μ l). The gel was scanned with LAS-1000 reader (Fuji, Tokyo, Japan) and analyzed with AIDA 2.11 software (Isotopenmessgeraete, Straubenhardt, Germany). The point of equivalence between the native and the competitor RNAs was computed using the PCRFIT program (Vu et al., 2000) (Fig. 1).

Results

Data summarized in Table 2 show that the concentration of mRNA for the M₂ subtype of muscarinic receptors vastly exceeded the concentrations of mRNAs for all non-M₂ subtypes in the investigated three parts of the heart. Expressed as a percentage of the sum of mRNAs for all muscarinic receptor subtypes taken together, the M₂ mRNA represented 92% in the left ventricle, 93% in the right ventricle, and 94% in the atria.

There were more than twice as many M₂ mRNA molecules per 1 μ g of total RNA in the atria than in the ventricles, and the total concentration of mRNA molecules for muscarinic receptors was also more than twice as high in the atria compared with the ventricles (Table 2). The M₁, M₄, and M₅ mRNAs seemed more abundant in the atria than in ventricles similarly to the M₂ mRNA, but it was only in the case of the M₁ mRNA that the difference between the atria and ventricles reached statistical significance. The M₃ mRNA seemed to be uniformly distributed.

The concentrations of M₂ mRNA that we discovered were remarkably close to those discovered in rat atria and ventricles by Hardouin et al. (1998): nearly 9×10^6 molecules per 1 μ g of total RNA in the atria and 4×10^6 molecules per 1 μ g of total RNA in the ventricles. To ensure that the substantially lower concentrations of the non-M₂ mRNAs we found (compared with the M₂ mRNA) were not caused by unsuitable primers or another failure of the RT-PCR reaction, we performed two types of control experiments: (1) we quantitated the M₁ mRNA with two different sets of primers (see *Materials and Methods*) and obtained identical results, which have been pooled in Table 1; and (2) we performed the quantitation of the M₁ to M₅ mRNAs in rat brain cortex (Table 3). The concentrations of mRNAs for individual muscarinic receptor subtypes and the quantitative proportions between subtypes were substantially different in the brain cortex than in the heart, but they were in accord with what is known about the distribution of the M₁ to M₅ muscarinic receptor

TABLE 2

Concentrations and relative representation of mRNAs for muscarinic M₁ to M₅ receptor subtypes in rat cardiac atria and right and left ventricles

Data indicate the numbers of mRNA molecules for individual muscarinic receptor subtypes (mean \pm S.E. of *n* determinations) per 1 μ g of total RNA extracted from the respective part of the heart, and the proportions (percentage) that mRNAs for individual subtypes represent of the sum of mRNAs for all subtypes of muscarinic receptors taken together.

| | M ₁ | M ₂ | M ₃ | M ₄ | M ₅ | Total M ₁ –M ₅ |
|-----------------|--------------------------------------|------------------------------|---------------------------------------|-------------------------------------|-----------------------------------------|--------------------------------------|
| Atria | | | | | | |
| Concentration | 60,960 ^a \pm 15,510 (7) | 7,526,000 \pm 965,300 (10) | 90,650 ^a \pm 22,170 (6) | 15,940 ^a \pm 5,496 (4) | 324,800 ^{a,b} \pm 80,210 (6) | 8,018,350 |
| Percentage | 0.8 | 93.9 | 1.1 | 0.2 | 4.1 | 100 |
| Right Ventricle | | | | | | |
| Concentration | 651 ^a \pm 261 | 3,421,000 \pm 629,100 (9) | 109,400 ^a \pm 38,600 (5) | 3,038 ^a \pm 335 (4) | 195,000 ^{a,b} \pm 60,770 (6) | 3,729,095 |
| Percentage | 0.02 | 91.7 | 2.9 | 0.1 | 5.2 | 100 |
| Left Ventricle | | | | | | |
| Concentration | 417 ^a \pm 53 | 3,057,000 \pm 557,700 (7) | 90,880 ^a \pm 32,990 (5) | 4,233 ^a \pm 1,595 (4) | 120,300 ^{a,b} \pm 38,450 (6) | 3,272,830 |
| Percentage | 0.01 | 93.4 | 2.8 | 0.1 | 3.7 | 100 |

^a Significantly different from M₂ in the same part of the heart (analysis of variance followed by Newman-Keuls multiple comparison test).

^b Significantly different from M₁, M₃, and M₄ in the same part of the heart if the analysis of variance is applied solely to data on the non-M₂ subtypes.

protein in the brain. The results obtained with cerebrocortical M_1 to M_5 mRNAs thus indicated that the low concentrations of the non- M_2 mRNAs we detected in the heart were not caused by an inability of our method to appropriately detect the non- M_2 subtypes.

Discussion

The most important findings we have described are (1) that the M_2 mRNAs represent the majority (>90%) of total muscarinic mRNAs in both the atria and the ventricles of the rat heart, and (2) that with a sufficiently sensitive method, mRNAs for all non- M_2 muscarinic receptor subtypes can be detected in both the atria and the ventricles, but that their contribution to the total pool of muscarinic mRNAs is minor. Our data thus substantiate the original concept of the dominant position of the M_2 receptors in mammalian heart. Recently, this concept received strong support on the functional level from the discovery that the bradycardic effect of carbamylcholine was completely lost in the M_2 receptor knockout mice (Stengel et al., 2000). Despite original contradic-

tions, the M_2 subtype has also been found to represent the fully prevailing subtype (evaluated according to the expression of mRNAs) in the heart of chicks (McKinnon and Nathanson, 1995).

Our data do not permit us to differentiate between the mRNAs present in cardiomyocytes, intrinsic neural tissue, blood vessels, and nonmyogenic cells of the heart. The density of neural elements is substantially higher in rat atria than in their ventricles, and the 100-fold higher concentration of the M_1 mRNA in the atria compared with the ventricles perhaps reflects this difference. This leads to the assumption that the M_1 mRNA is located in neurons, not in cardiomyocytes. Such an assumption agrees with reports that, through in situ hybridization, M_1 mRNA could be detected in cardiac intrinsic ganglia but not in atrial myocytes or ventricular muscle (Hassall et al., 1993; Hoover et al., 1994), and with functional data concerning the M_1 receptors in autonomic ganglia (Caulfield, 1993). However, the assumption is at variance with the report that, by the use of single-cell RT-PCR, the M_1 mRNA could be detected in cultured ventricular myocytes (Colecraft et al., 1998).

TABLE 3
 Concentrations and relative representation of mRNAs for muscarinic M_1 to M_5 receptor subtypes in rat brain cortex with subcortical white matter
 Data indicate the numbers of mRNA molecules for individual muscarinic receptor subtypes (mean \pm S.E. of three determinations) per 1 ng of total cerebrocortical RNA, and the proportions (percentage) that mRNAs for individual subtypes represent of the sum of mRNAs for all subtypes of muscarinic receptors taken together.

| | M_1 | M_2 | M_3 | M_4 | M_5 | Total M_1 - M_5 |
|---------------|--------------------|--------------------|--------------------|-------------------|-------------------|---------------------|
| Concentration | 17,600 \pm 3,496 | 10,470 \pm 2,401 | 12,500 \pm 3,617 | 5,197 \pm 1,242 | 3,391 \pm 1,399 | 49,158 |
| Percentage | 35.8 | 21.3 | 25.4 | 10.6 | 6.9 | 100 |

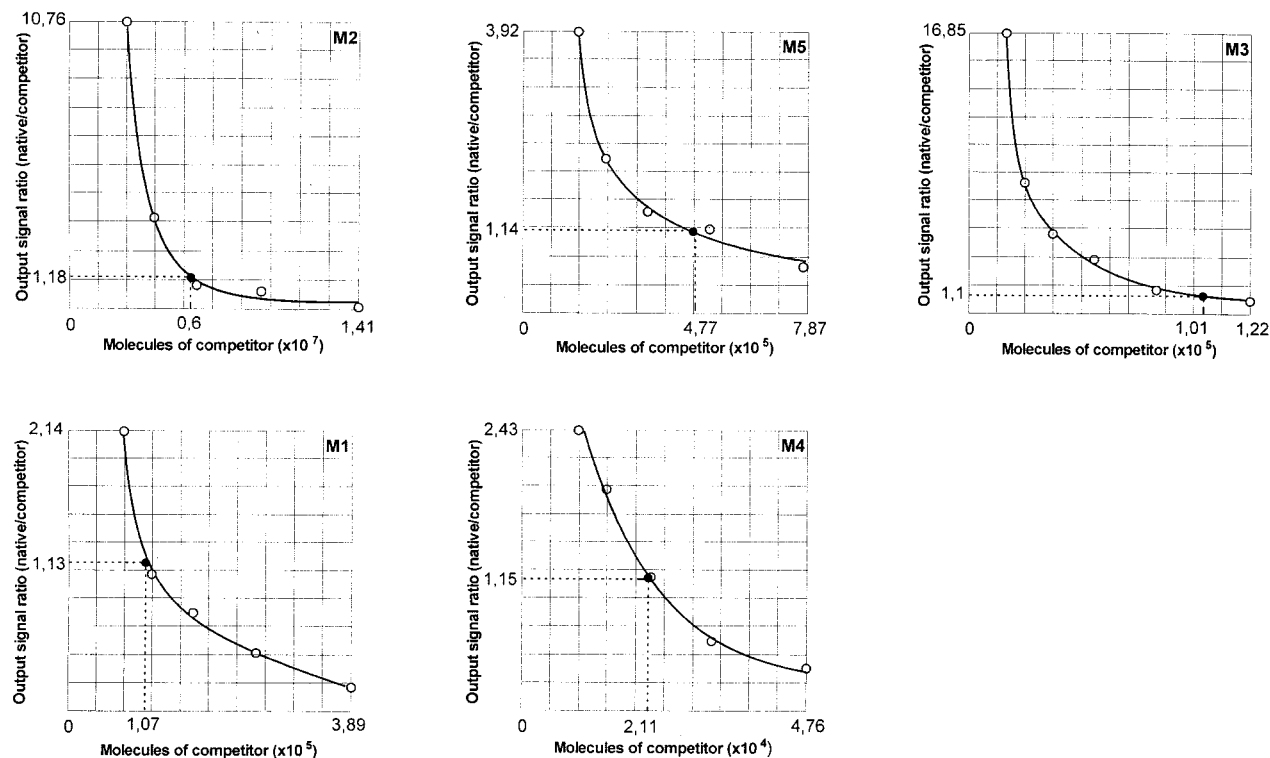


Fig. 1. Representative curves from individual determinations of the concentrations of mRNAs for the M_1 to M_5 muscarinic receptor subtypes by competitive RT-PCR. x-axis, number of competitor molecules added to samples; y-axis, ratio of fluorimetric intensities (native cDNA/competitor cDNA) as determined in gels after reverse transcription and PCR amplification. Broken lines, points of equivalence, where the number of competitor molecules equals the number of native cDNA molecules. At the point of equivalence, the ratio of fluorimetric intensities equals the ratio (length of native cDNA)/(length of competitor cDNA). Curves have been drawn using a computer to fit polynomial equations that determine the concentration ratio (native cDNA)/(competitor cDNA) after n cycles of PCR (eqs. 5 and 6 in Vu et al., 2000). \circ , actual data points. \bullet , computed points of equivalence.

In contrast to all other subtypes, the M_3 mRNA seemed to be equally concentrated in the atria and the ventricles. This suggests that its location is non-neural, but it is difficult to decide with the present knowledge whether it is located in cardiomyocytes or in nonmyogenic cardiac cells. At least a proportion of the M_3 mRNA (if not all of it) would be expected to derive from the smooth muscle cells of the blood vessels (Eglen et al., 1996; Phillips et al., 1997). Pharmacological data have been published that suggest the presence of M_3 receptor protein in cardiocytes from newborn rats (Yang et al., 1993; Sun et al., 1996).

Of the other non- M_2 mRNAs, the M_4 mRNA is clearly little expressed in the heart. A noteworthy feature of our data is that they point to the M_5 subtype as the most strongly expressed non- M_2 muscarinic receptor subtype in both the atria and the ventricles. Unfortunately, the M_5 subtype is the most difficult muscarinic receptor subtype to detect with approaches using radiolabeled ligands. Should the relative concentration of the M_5 receptor protein correspond to the relative concentration of the M_5 mRNA (i.e., 4–5% of total muscarinic receptors), it would be difficult to detect the M_5 receptor population using methods available currently, although its identification and the clarification of its role in the heart are a serious challenge. Cellular location of the M_5 mRNA in the heart is unknown. Hassall et al. (1993) could not detect the M_5 mRNA in intrinsic cardiac ganglia using *in situ* hybridization. Reports on the presence of M_5 mRNA in rat arteries (Phillips et al., 1997), human brain microvascular endothelial cells (Elhousseiny et al., 1999), and human skin fibroblasts (Buchli et al., 1999) suggest that cardiac endothelial cells and fibroblasts should be considered among potential sources of the M_5 mRNA we detected in the heart.

As mentioned under *Results*, there is excellent agreement between our data on cardiac M_2 mRNA and those of Hardouin et al. (1998). Data on the concentrations of mRNAs for the other subtypes of cardiac muscarinic receptors are not available. The concentrations of mRNAs for all subtypes of muscarinic receptors were higher in cerebral cortex than in the heart, which is in harmony with the much higher density of muscarinic binding sites in the brain cortex (Nedoma et al., 1986; Ehlerl and Tran, 1990). To our knowledge, our data on mRNAs in cerebral cortex are the first to permit direct comparison of the concentrations of mRNAs for individual subtypes of muscarinic receptors in this part of the brain. Subtype-selective immunoprecipitation of receptor protein (Tice et al., 1996) indicated a lower proportion of the M_3 and a higher proportion of the M_4 subtype than our data on corresponding mRNAs.

The quantitative data we described should facilitate the evaluation of the likely roles of individual subtypes of muscarinic receptors both in the heart and in the brain cortex. They should also facilitate the studies of how their expression is controlled and assist in attempts to identify cardiac non- M_2 muscarinic receptor subtypes at the protein level (Shi et al., 1999; Mysliveček et al., 2001).

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