0026-895X/01/5905-1029–1036$3.00
MOLECULAR PHARMACOLOGY
Copyright © 2001 The American Society for Pharmacology and Experimental Therapeutics
Mol Pharmacol 59:1029–1036, 2001

Vol. 59, No. 5
512/898173
Printed in U.S.A.

Expression of Multiple Subtypes of Muscarinic Receptors and Cellular Distribution in the Human Heart

HUIZHEN WANG, HONG HAN, LIMING ZHANG, HONG SHI, GERNOT SCHRAM, STANLEY NATTEL, and ZHIGUO WANG

Research Center, Montreal Heart Institute, Montreal, Quebec, Canada (H.W., H.H., L.Z., H.S., G.S., S.N., Z.W.); Department of Medicine, Montreal Heart Institute and University of Montreal, Montreal, Quebec, Canada (S.N., Z.W.); and Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec, Canada (S.N.)

Received August 22, 2000; accepted January 18, 2001

This paper is available online at http://molpharm.aspetjournals.org

ABSTRACT

Five isoforms of the muscarinic acetylcholine receptor (mAChR) have been identified by molecular cloning and designated m_1–m_5, of which four correspond to the functional subtypes M_1, M_2, M_3, and M_4 in primary tissues. The presence of M_5 receptors in tissues remains uncertain. The present study was designed to explore the diversity and cellular distribution of various mAChR subtypes in human hearts. Competition binding of [*N*-methyl-^{3}H]-scopolamine methyl chloride with various mAChR antagonists yielded data consistent with the presence of multiple subtypes (M_1/M_2/M_3/M_5) of mAChRs in both human atrial (HA) and ventricular (HV) tissues. Expression of mRNAs encoding all five subtypes was readily detected by reverse transcription-polymerase chain reaction in both HA and HV samples. Immunoblotting with subtype-specific antibodies confirmed the presence of M_1, M_2, M_3, and M_5, but not M_4, proteins in membrane preparations from both HA and HV. The protein levels of M_1 and M_2 were comparable between HA and HV. Although the density of M_3 appeared ~10-fold higher in HV than HA, that of M_5 was ~5 times lower in HV than in HA. Positive immunostaining of single ventricular myocytes by M_1, M_2, M_3, and M_5 antibodies, respectively, was consistently detected. Under confocal microscopy, M_5 showed characteristic localization to the intercalated discs, whereas other subtypes were more evenly distributed throughout the surface membrane. Our results provide the first molecular evidence for the presence of multiple subtypes of mAChR, including endogenous M_5 receptors, in human hearts and suggest that different subtypes have different tissue distributions and cellular localization.

Not until the 1990s were multiple subtypes (M_1, M_2, M_3, and M_4) of mAChRs functionally defined in primary tissues with the development of pharmacological probes (Doods et al., 1987; Mitchelson, 1988; Goyal, 1989; Mutschler et al., 1989; Hulme et al., 1990; van Zwieten and Doods, 1995; Eglen and Watson, 1996). cDNAs representing five different isoforms of mAChR subunits, m_1 through m_5, have been cloned from a variety of mammals, including humans (Bonner et al., 1987; Peralta et al., 1987; Goyal, 1989; Brann et al., 1993). M_1–M_4 receptors seem to correspond to the cloned m_1–m_4 isoforms, whereas the physiological counterpart of m_5 is yet to be established.

This work was supported in part by the Medical Research Council of Canada, the Heart and Stroke Foundation of Quebec, an Establishment Grant for young investigators from the Fonds de Recherche en Santé du Québec awarded to Dr. Wang, and the Fonds de Recherche de l'Institut de Cardiologie de Montréal. Z.W. is a Research Scholar of the Heart and Stroke Foundation of Canada. H.W. and H.S. are Research Fellows of the Medical Research Council of Canada. H.H. is a Research Fellow of the Heart and Stroke Foundation of Canada.

Muscarinic receptor stimulation by acetylcholine plays an important role in mediating parasympathetic control of cardiac functions such because heart rate, conduction, and contractility. In contrast to most peripheral tissues, the myocardium has generally been considered to possess a single mAChR subtype: M_2 receptors have long been believed to be the only functional mAChR subtype in the heart (Bonner et al., 1987; Peralta et al., 1987; Dörje et al., 1991; Pappano, 1991). In fact, the heart is commonly used because a model illustrating the exclusive presence of M_2 receptors. However, the concept that the heart possesses a single M_2 subtype of mAChR is now being challenged. Many physiological responses to mAChR stimulation cannot be explained on the basis of a single M_2 subtype in the heart. Accumulating evidence suggests the existence of mAChR subtypes other than M_2 in cardiac tissues of chickens, rats, guinea pigs, rabbits, and dogs (Jaiswal et al., 1989; Akahane et al., 1990; Tietje and Nathanson, 1991; Ford, 1992; Yang et al., 1992; Gadbut and Galper, 1994; Kan et al., 1996; Sharma et al.,

ABBREVIATIONS: mAChR, muscarinic acetylcholine receptor; RT, reverse transcription; PCR, polymerase chain reaction; HA, human atrium; HV, human ventricle; PBS, phosphate-buffered saline; [^{3}H]NMS, [*N*-methyl-^{3}H]scopolamine methyl chloride; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodide; bp, base pair(s); HHSiD, hexahydro-siladifenidol.

1996, 1997; Sun et al., 1996), with large variations of subtype expression among species.

The cDNA of the m_5 muscarinic receptor was first cloned over a decade ago. In artificial expression systems, m_5 receptors have been shown to couple to multiple signaling mechanisms, including stimulation of phospholipase C-protein kinase C, phospholipase A_2-arachidonic acid, and mitogen-activated protein kinase; inhibition of cAMP production; activation of nitric-oxide synthase; and intracellular calcium mobilization (Kohn et al., 1996; Reever et al., 1997; Kukkonen et al., 1998; Wotta et al., 1998). The physiological significance of the m_5 subtype remains a mystery because of a lack of m_5-selective ligands and a lack of evidence that primary tissues express the M_5 receptor. Only recently has the presence of native M_5 receptors in the brain been suggested by pharmacological approaches (Reever et al., 1997).

We recently reported functional and molecular evidence for the presence of M_3 and M_4 receptors in canine (Shi et al., 1999a,b; Wang et al., 1999b) and M_3 receptors in guinea pig atrial myocytes (Shi et al., 1999b; Wang et al., 1999b). We found these receptors to be functionally coupled to two novel and distinct K^+ channels. Activation of M_3 receptors in guinea pig atrial preparations promotes membrane repolarization and slows sinus rate (Shi et al., 1999b; Wang et al., 1999b). Whether the human heart possesses mAChRs other than M_2 remains unknown. To date, no systematic studies have examined mAChR subtypes in the human heart. We therefore examined the expression of various mAChR subtypes in human hearts by analyzing pharmacological properties (radioligand binding displacement), mRNA expression (RT-PCR), tissue protein concentrations (Western blot), and cellular protein distribution (immunostaining/confocal microscopy).

Materials and Methods

Membrane Protein Preparation. Specimens of human right atrial appendage (HA) were obtained from the hearts of four patients undergoing aortocoronary bypass surgery. The atria were from patients without heart failure, atrial arrhythmias, or electrocardiographic P-wave abnormalities and were grossly normal in appearance. Left ventricular tissues (HV) were dissected from the explanted hearts of four patients (aged 57–71) receiving heart transplantations. The procedure for obtaining the tissue was approved by the Ethics Committee of the Montreal Heart Institute.

The procedures for membrane protein preparation have been described previously (Shi et al., 1999a,b; Wang et al., 1999b). The preparations were minced and washed with ice-cold phosphate-buffered saline (PBS) buffer. The tissues were then homogenized with a Polytron in 15 ml of ice-cold lysis buffer containing 5 mM Tris-HCl, 2 mM EDTA, pH 7.4, and a protease inhibitor cocktail consisting of 5 mg/ml phenylmethylsulfonyl fluoride, 10 mg/ml benzamidine, and 5 mg/ml soybean trypsin inhibitor. The homogenate was centrifuged at 500*g* for 15 min at 4°C. The pellets were then homogenized as before, spun again, and the supernatants pooled. The supernatants were centrifuged at 45,000*g* for 15 min and the pellets washed twice in the same buffer. The membrane fractions were resuspended in a buffer containing 75 mM Tris-HCl, pH 7.4, 12. 5 mM $MgCl_2$, and 5 mM EDTA. The protein content was determined with Bio-Rad Protein Assay kit (Bio-Rad, Mississauga, ON, Canada) using bovine serum albumin as the standard. Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Membrane Receptor Binding Assay. Saturation binding assays were performed using eight concentrations of [*N*-methyl-^{3}H]scopolamine methyl chloride ([^{3}H]NMS, 82 Ci/mmol) ranging from 2 to 2500 pM. Binding measurements were obtained in triplicate for each experiment with total of eight individual preparations (from four atria and four ventricles). Incubations at a volume of 1 ml (90 min at room temperature) were terminated by rapid filtration with Whatman GF/C filters (Xymotech, Montreal, PQ, Canada), and radioactivity was counted with an LS6500 Scintillation Counter (Beckman, Fullerton, CA). Nonspecific binding was defined as that measured in the presence of 1 μM atropine. Specific binding (averaging ~90% of total binding) was determined by subtracting nonspecific from total binding (Shi et al., 1999a,b; Wang et al., 1999).

Competition binding assays were carried out as follows (Shi et al., 1999a,b; Wang et al., 1999b): Homogenates were incubated with 400 pM [^{3}H]NMS and pirenzepine (0.3 nM–100 μM), methoctramine (0.3 nM–100 mM), 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP, 0.1 nM–100 μM), or tropicamide (1 nM–1 μM), respectively. In addition, choline chloride (10 nM–10 mM) and tetramethylammonium (30 nM–1 mM), which have been shown to activate M_3 receptors in canine myocardium (Shi et al., 1999a,b), were also studied in competition binding experiments. A fixed amount of membrane protein (100 μg) was used for each binding study. Reagents for binding experiments were purchased from RBI/Sigma (Natick, MA).

RT-PCR. RNA was isolated as described previously (Wang et al., 1998, 1999a; Shi et al., 1999; Yue et al., 1999). RNA samples were isolated from adjacent tissues of the same hearts used for membrane protein extraction. Total RNA samples extracted from human atrial and ventricular tissues were incubated with DNase I (0.1 units/ml) at 37°C for 15 min, and this was followed by phenol/chloroform extraction to remove genomic DNA.

Reverse transcription was carried out in a 20-μl reaction mixture containing 1× reaction buffer [10 mM Tris-HCl, pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$], 1 mM dNTPs (Roche Molecular Biochemicals, Montreal, PQ, Canada), 3.2 mg of random primers $p(dN)_6$ (Roche Molecular Biochemicals), 5 mM dithiothreitol, 50 units of RNase inhibitor (Canadian Life Technologies, Burlington, ON, Canada), and 200 units of Moloney murine leukemia virus reverse transcriptase (Life Technologies). First-strand cDNA was synthesized at 42°C for 60 min, and remaining enzymes were heat-inactivated at 99°C for 5 min. First-strand cDNA (5 μl) was used as a template for amplification in a 25-μl reaction mixture. Reagents included in each reaction were 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 1 mM dNTPs, 0.5 μM each gene-specific primer, and 2.5 units of *Taq* polymerase (Life Technologies). Reactions were hot-started at 94°C and continued for 3 min of initial melting. The cycling profiles were 30-s denaturing at 94°C, 30-s annealing at 52°C, and 40-s extension at 72°C, for 30 cycles, followed by a final extension step of 5 min at 72°C. The following primers were synthesized by Life Technologies: M_1 isoform, agactcctccaggccgctgc (sense) and ctcctttccacggggcttctg (antisense); M_2 isoform, aagaaggacaagaaggagcc (sense) and ctttggaatggcccagg (antisense); M_3 isoform, tggaacaacaatgatgctgc (sense) and ccttttccgcttagtgatctg (antisense); M_4 isoform, cccgaaggagaagaaagc (sense) and agtggtggcctctgtgg (antisense); and M_5 isoform, atcatgccctgccccttcc (sense) and gtagcttgctgttcccctgcc (antisense). All primers were designed to avoid significant secondary and complementary structures and to contain about 50 to 60% G-C content. The gene-specific primer pairs were designed based on cDNA sequences of human mAChRs (Peralta et al., 1987; Bonner et al., 1988), and unique oligonucleotide sequences were chosen from cDNA regions with minimal homology among different mAChR isoforms. The specificity of primer pairs was verified by comparison with the entire GenBank database using BLAST and by amplification of a PCR product of the predicted size visible as a single discrete band with ethidium bromide staining on an agarose gel.

Western Blot Analysis. Membrane proteins (60 μg) were fractionated by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide gels) and transferred to nitrocellulose membranes (Immobilon-P, pore size 0.45 mm; Millipore Corporation, Bedford, MA) in a transfer buffer (25 mM Tris-base, 192 mM glycine, 20% methanol,

and 0.01% SDS) (Wang et al., 1999a; Yue et al., 1999). After transfer, the membrane was washed in Tris-buffered saline (Tris-HCl, NaCl, distilled H_2O, pH 7.5) with 0.05% Tween 20 for 10 min and then incubated in a blocking buffer containing 5% nonfat dry milk in 0.1% Tris-buffer saline/Tween 20 (Tris-buffered + 0.1% Tween 20) for 2 h, followed by overnight incubation at 4°C with the primary antibody. The M_1, M_2, M_3, and M_5 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and the M_4 antibody from Chemicon International (Temecula, CA). The next day, the membrane was washed three times in 0.1% Tris-buffered saline/Tween 20 (10 min/wash) and incubated for 2 h with the secondary antibody (avidin-horseradish peroxidase-conjugated goat anti-mouse IgG for M_4 and goat anti-rabbit IgG for other subtypes) in the blocking buffer. The membrane was then washed in the blocking buffer for 15 min. Bound antibodies were detected using chemiluminescent substrate (Western Blot Chemiluminescence Reagent Plus; PerkinElmer Life Science Products, Boston, MA). A rat brain protein sample (20 μg) was used as a positive control for mAChRs and negative controls were performed by preincubating the antibodies with the respective peptides against which they are generated. The presence of a given subtype of mAChR was verified by the presence of a prominent band with molecular mass in the range of previous reports and by elimination of the band in preparations preincubated with the antigenic peptide. Potential cross-reaction of antibodies was excluded using purified M_1, M_2, M_3, and M_5 receptors obtained from Santa Cruz Biotechnology. Coomassie staining was performed to verify the amount of protein inputs by incubate the membranes in Coomassie Brilliant Blue (prepared as a working solution in 50% methanol and 10% acetic acid solution) for 2 min and then washed to remove the background. Experiments were discarded if any visible differences between samples for comparison (HA versus HV) were found.

Immunocytochemistry. Human ventricular myocytes were isolated from the explanted hearts of three patients receiving heart transplantation, using methods described previously in detail (Li et al., 1995). A segment of the left ventricular free wall containing a coronary artery was perfused via a Langendorff-type system with Ca^{2+}-containing Tyrode's solution at 37°C until the effluent was clear of blood. The perfusate was then changed to Ca^{2+}-free Tyrode's solution for 20 min at 12 ml/min, followed by perfusion with the same solution containing collagenase (110 U/ml CLS-II collagenase; Worthington Biochemical, Freehold, NJ) and 0.1% bovine serum albumin (Sigma Chemical Co., St. Louis, MO). The Tyrode's solution contained 136 mM NaCl, 5.4 mM KCl, 1 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 5 mM HEPES, 10 mM glucose, and 1 mM $CaCl_2$; pH was adjusted to 7.4 with NaOH.

The dispersed cells were washed twice with fresh PBS and plated on laminin (15 μg/ml)-coated coverslips in the wells of culture dishes containing Dulbecco's modified Eagle's medium, supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (10 mg/ml penicillin and 1 mg/ml streptomycin). Cells were incubated at 37°C for 1 h. The culture medium was then discarded and the cells were incubated with 1 ml of paraformaldehyde (1%, pH 7.4) for 30 min. After two washes with PBS, the cells were treated with Triton (1%) for 5 min to permeabilize cell membranes and then incubated overnight with 1% bovine serum albumin at 4°C. The following day, the cells were exposed to primary anti-mAChR antibodies for 2 h. After a PBS wash, the cells were incubated in secondary antibody for 1 h. Coverslips were then placed onto glass slides and sealed with mounting medium for confocal microscopy.

Data Analysis. Group data are expressed as the mean ± standard error. Binding data were analyzed using curve-fitting functions in GraphPad Prism software (GraphPad Software, San Diego, CA). Linear regression was performed on the percentage of bound versus the ratio of bound over free ligand, and only data with a regression coefficient of ≥0.9 were used for analyses. The F test was used to compare fits for the competition binding data, and the best fit (one-site binding versus two-site binding) was determined by the probability value for the F test and by the change in the residual sum of squares for the two different fits. One- and two-site models were tested for all data sets, and the model yielding the least residual sum of squares was taken to describe the data. Densities of the immunoblot bands were quantified with the use of a Molecular Imager System (GS-505; Bio-Rad).

Results

Pharmacological Identification of Multiple Subtypes of mAChR in Human Hearts. [^{3}H]NMS binding to membrane homogenates from human atrial and ventricular tissues was saturable over the concentration range (2–2500 pM) examined. As illustrated in Fig. 1, the experimental data were well fitted by a one-site binding model. Mean K_d values were 220 ± 20 pM for HA and 259 ± 29 pM for HV, and the B_{max} value was 280 ± 40 fmol/mg protein for HA and 259 ± 51 fmol/mg protein for HV (n = 4/group). A Scatchard analysis of saturation binding data (Fig. 1A, inset) was linear, consistent with binding to a single class of receptor sites.

Competition binding studies were performed between [^{3}H]NMS (400 pM) and the following selective antagonists: pirenzepine (M_1-selective) (Watson et al., 1983; van Zwieten and Doods, 1995), methoctramine (M_2-selective) (Michel and Whiting, 1988; van Zwieten and Doods, 1995), 4-DAMP (M_3-selective) (Barlow and Shepherd, 1986; Michel et al., 1989; Araujo et al., 1991; van Zwieten and Doods, 1995), and tropicamide (M_4-selective) (Lazareno et al., 1990; Lazareno and Birdsall, 1993). The data from each curve gave the best fit to a two-site binding model (Fig. 1). Table 1 provides pK_i values, together with the percentage of total binding represented by

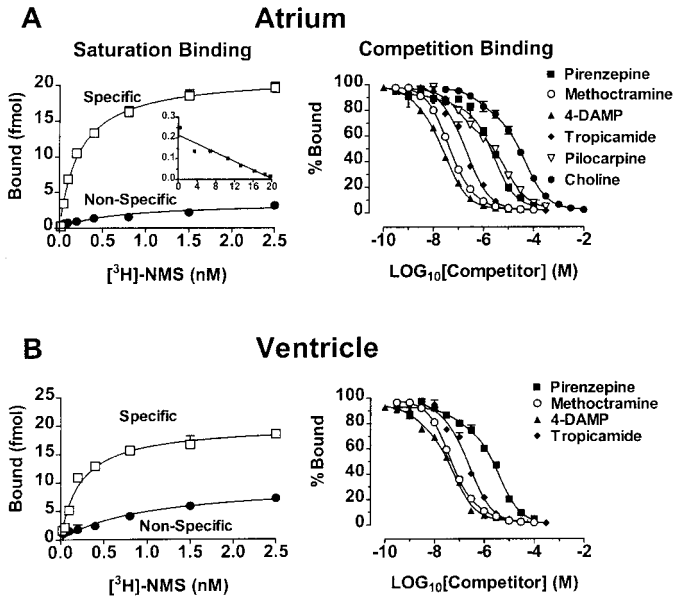

Fig. 1. Binding of [^{3}H]NMS to homogenates of human atrial (A) and ventricular (B) tissues. Left, saturation binding of [^{3}H]NMS. Symbols are experimental data (mean ± S.E.M.) and lines represent fits by a one-site binding model. □, specific binding; ●, nonspecific binding obtained in the presence of atropine (1 μM). Shown in the inset of A is the Scatchard plot of the atrial data, with the regression line to the data points shown [the title for the y-axis, Bound/Free, and for the x-axis, Bound (fmol)]. Data shown are averaged from four individual preparations carried out in triplicate for each experiment. B, competition binding of [^{3}H]NMS with various compounds. Each data point represents mean ± S.E. from six experiments (six hearts) assayed in duplicate. Curves are best fits of the experimental data to a two-site binding model.

the high-affinity site for each antagonist. Comparison with previously published values of the relative affinities for these antagonists (Doods et al., 1987; Mitchelson, 1988; Goyal, 1989; Mutschler et al., 1989; Hulme et al., 1990; Lazareno et al., 1990; Lazareno and Birdsall, 1993; van Zwieten and Doods, 1995; Eglen and Watson, 1996) suggests the presence of multiple mAChR subtypes ($M_1/M_2/M_3/M_5$).

Expression of mRNAs Encoding Different mAChR Isoforms in Human Hearts. RT-PCR showed the presence of mRNA corresponding to all five mAChR isoforms (m_1–m_5) in HA (Fig. 2A). Note bands at the expected molecular weights for m_1 [275 base pairs (bp), lane 1], m_2 (297 bp, lane 3), m_3 (432 bp, lane 5), m_4 (257 bp, lane 7), and m_5 (391 bp, lane 9). Similar results were obtained from the other three mRNA samples from HA and from a total of three HV samples. Note also the absence of bands in RT-negative controls (laness 2, 4, 6, 8, and 10).

To evaluate possible contamination of sample RNA by noncardiac tissue, we performed additional experiments investigating RT-PCR amplification of the neuronal acetylcholine β_4 subunit (for neural contamination) and the maxi-K channel (Ca^{2+}-activated K^+ channel, for vascular contamination) (Wang et al., 1998, 1999a). Primers for β_4 and maxi-K detected strong signals in RNA samples extracted from rat brain and rat vascular smooth muscle, respectively (Fig. 2B). The absence of corresponding signals with cardiac RNA samples excludes significant neural and vascular tissue contamination of cardiac samples (Dixon and McKinnon, 1994; Wang et al., 1998).

Immunoblotting of mAChR Proteins in Human Hearts. To clarify whether multiple mAChR subtypes also express at the protein level in human hearts, immunoblotting analysis was performed with fractionated HA and HV membrane proteins. Western blots with the anti-m_1, m_2, m_3, or m_5 antibody revealed prominent protein bands at ≈100, 84, 113, and 80 kDa, respectively (Fig. 3A). The bands were eliminated when the antibodies were preincubated with the respective peptides against which they were generated. Similar results were consistently obtained with all four preparations from different patients. The presence of proteins representing M_1, M_2, M_3, and M_5 receptors were also consistently revealed in all three HV samples with band sizes identical to the respective subtypes found in the HA preparations. M_4 receptors were detected neither in HA nor in HV, even with increased quantities of both antibody and membrane preparation (up to 150 μg/reaction). In contrast, the anti-m_4 antibody labeled a clear band representing M_4 receptors in the rat brain preparation, the positive control. For $M_{1–3}$ and M_5 receptors, the band sizes of the positive (rat brain) controls were in the same range as for the corresponding subtypes in the human heart.

A fixed amount of membrane protein (60 μg) was used for each Western blot determination and the equal input of different protein samples was verified by Coomassie staining (No visible differences between HA and HV samples were found.). The densities of M_1 and M_2 receptors was equivalent in HA versus HV (1:1 HA:HV for M_1 and 1.2:1 for M_2). However, the expression of M_3 protein seemed higher in the HA, whereas that of M_5 was lower in HA, compared with HV, with HA:HV band density ratios of ~1:0.1 for M_3 and 0.2:1 for M_5.

We considered the possibility that the bands identified in our experiments were due to cross-reaction with other mAChR subtypes. The subtype specificities of the antibodies have been tested and certified by the manufacturer (Santa Cruz Biotechnology). Nevertheless, to address the possibility further, we incubated pure preparations of expressed human M_1, M_2, M_3, and M_5 receptors (RBI/Sigma) with each of the antibodies. No cross-reactivity was found for any of the five antibodies used in this study (Fig. 3B); each antibody identified a single band for the appropriate receptor, with a size similar to the corresponding band in human membrane proteins.

Immunostaining of mAChRs in Isolated Human Ventricular Myocytes. To investigate further the specific cellular localization of various mAChR subtypes in human heart cells, we performed immunocytochemistry with isolated human ventricular myocytes. Figure 4 presents typical examples of such experiments, showing the green fluorescence staining of various subtypes of mAChR under confocal microscopy. Consistent with Western blot studies, cells exposed to antibodies against M_1, M_2, M_3, and M_5 receptors showed clear sarcolemmal staining; whereas antibody against M_4 receptors failed to produce any positive signals. M_5 receptor staining seemed to be largely restricted to the intercalated discs, whereas other receptor subtypes were evenly distributed along the surface membrane. M_3 receptors also demonstrated stronger staining on the intercalated discs relative to other regions of the plasma membrane. Nonspecific and subtype cross-reactivities were excluded by the elimination of staining after pretreatment of the antibodies with their respective antigenic peptides.

TABLE 1

mAChR antagonist binding in HA and HV membrane homogenate

K_{iH} and K_{iL} represent high- and low-affinity dissociation constants; values in the parentheses indicate the percentage of high-affinity binding.

Ligand	HA		HV	
	pK_{iH}	pK_{iL}	pK_{iH}	pK_{iL}
	nM		*nM*	
Pirenzepine	8.4 ± 0.7 (28.7 ± 8.7%)	6.3 ± 0.1	8.6 ± 1.2 (30.3 ± 10.4%)	6.1 ± 0.8
Methoctramine	8.6 ± 0.3 (77.5 ± 6.6%)	6.9 ± 0.3	8.8 ± 0.7 (78.2 ± 9.8%)	6.7 ± 0.5
4-DAMP	9.2 ± 0.2 (22.1 ± 4.2%)	7.9 ± 0.2	9.1 ± 0.4 (15.4 ± 2.5%)	7.7 ± 0.3
Tropicamide	7.4 ± 0.4 (20.5 ± 5.5%)	6.2 ± 0.2	7.3 ± 0.8 (18.7 ± 8.4%)	6.2 ± 0.8

Discussion

In the present study, we obtained several lines of evidence suggesting that multiple subtypes ($M_1/M_2/M_3/M_5$) of mAChR coexist in human hearts. Our results provide the first evidence for the presence of the endogenous M_5 receptor in mammalian hearts and suggest that different mAChR subtypes have distinct distributions in HA versus HV and characteristic cellular localizations.

Evidence for the Presence of Multiple Subtypes of mAChR in Human Hearts. The results from our competition binding experiments suggest that besides the long-recognized M_2 receptor, M_1 and M_3 subtypes are also expressed in the membrane of human cardiac cells. At present, there is no selective M_5 receptor antagonist. Thus, the data from binding experiments neither confirm nor exclude the presence of M_5 receptors. The displacement of [^{3}H]NMS binding by pirenzepine in HA yielded pK_i values of 8.4 and 6.3 for high- and low-affinity binding, respectively (Table 2). The high-affinity pK_i (8.4) is consistent with the previously reported binding affinity of pirenzepine for M_1 receptors (≈8.0) (Brann et al., 1993; van Zwieten and Doods, 1995; Eglen and Watson, 1996) and the fractional binding with this affinity is 28.7%, indicting a significant participation of M_1 receptors (Watson et al., 1983; van Zwieten and Doods, 1995). On the other hand, the low-affinity binding (pK_i = 6.5) does not discriminate among subtypes. The high-affinity binding of methoctramine apparently confirms the existence of M_2 receptors (van Zwieten and Doods, 1995), and the low-affinity binding (pK_i = 6.9) identifies but does not distinguish M_1 and M_4 subpopulations. Similarly, 4-DAMP binding also revealed two groups of mAChRs, with high-affinity binding (pK_i = 9.2) consistent with 4-DAMP affinity to M_3 and M_1 receptors (van Zwieten and Doods, 1995) and low-affinity binding (pK_i =

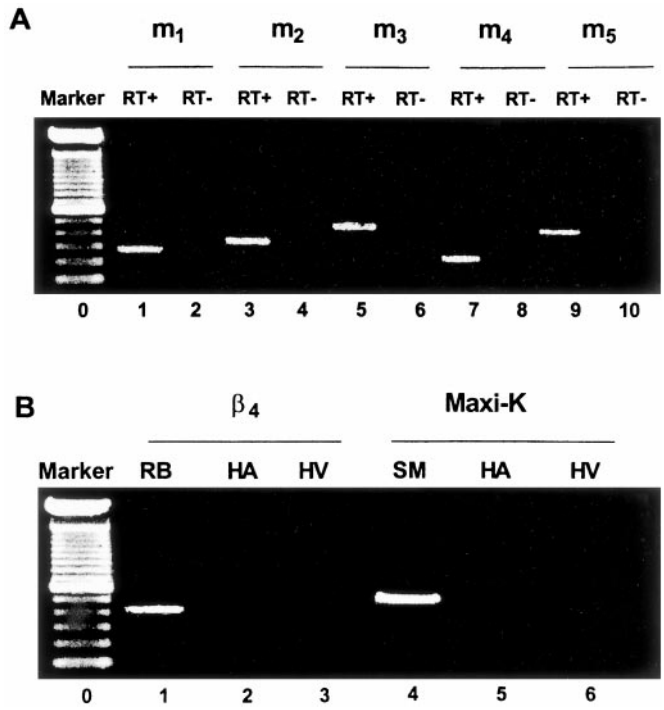

Fig. 2. Detection of mRNAs coding for various subtypes of mAChRs in mRNA samples purified from human atrial tissues. A, representative gel with ethidium bromide staining of PCR-amplified products. Lane 0, 100-bp cDNA size maker; lane 1 to 10, expected PCR products for M_1 (283-bp band), M_2 (297-bp band), M_3 (432-bp band), M_4 (257-bp band), and M_5 (391-bp band), respectively; RT+ indicates reaction with reverse transcriptase and RT− indicates omission of reverse transcriptase from the reaction to exclude the possible contamination by genomic DNAs. B, test for contamination by neuronal and vascular tissues. Lane 1, β_4 subunit of neuronal acetylcholine receptor with RNA from rat brain (411 bp), and lanes 2 and 3, human atrial and ventricular RNA, respectively, for detection of β_4 subunit; lane 6, Maxi-K channel with RNA from rat vascular smooth muscle (461 bp), and lanes 5 and lane 6, human atrial and ventricular RNA samples, respectively, for detection of Maxi-K.

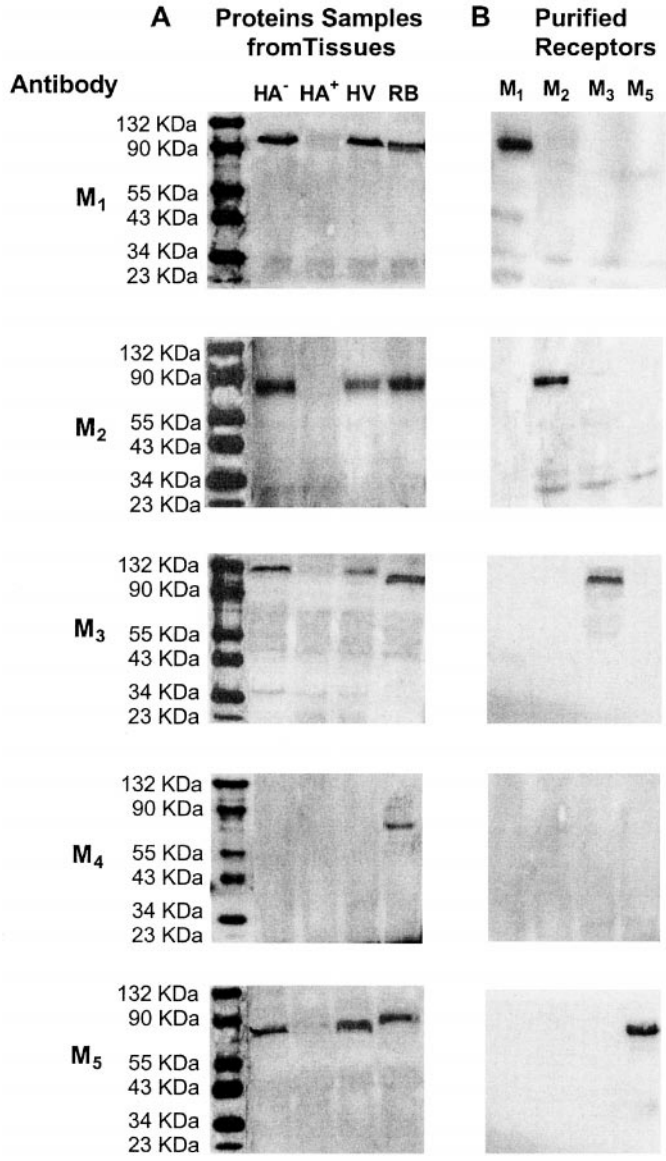

Fig. 3. A, Western blots of human atrial membrane protein with antibodies raised against specific subtypes of mAChR. The presence of various mAChR subtypes is revealed by bands with appropriate molecular masses identified by each subtype-specific antibody: M_1 ≈ 100 kDa, M_2 ≈ 84 kDa, M_3 ≈ 113 kDa, M_4 ≈ 75 kDa, and M_5 ≈ 80 kDa for human samples. The same results were obtained from three other atria and two HVs. For rat brain sample positive controls, the molecular masses are M_1 ≈ 97 kDa, M_2 ≈ 85 kDa, M_3 ≈ 102 kDa, M_4 ≈ 78 kDa, and M_5 ≈ 86 kDa. HA, human atrial protein exposed to antibody without preincubation with the antigenic peptide; HA^+, human atrial protein exposed to antibodies preincubated with their respective antigenic peptides; HV, human ventricular protein, and RB, rat brain protein. B, Western blots of purified M_1, M_2, M_3, and M_5 receptor proteins with antibodies raised against various subtypes of mAChR to test potential subtype cross-reactivity of antibodies. The molecular masses (band size) were M_1 ≈ 100 kDa, M_2 ≈ 84 kDa, M_3 ≈ 113 kDa, M_4 ≈ 75 kDa, and M_5 ≈ 80 kDa.

8.0) typical of 4-DAMP binding to M_2 receptors. Competition binding of tropicamide showed a high-affinity binding site (pK_i = 7.4), which fits well with the existence of M_3 and M_2 receptors (Lazareno et al., 1990; Lazareno and Birdsall, 1993). The low-affinity binding of tropicamide (pK_i = 6.2) does not fit with previously reported binding to a specific subtype. Taken together, the results from our binding experiments point to the existence of M_1, M_2, and M_3 receptors in HA. Similar results were obtained from HV preparations. One important weakness of using receptor antagonists is a lack of perfect specificity toward different subtypes, although the compounds used in our study represent the best choices available. Therefore, caution must be taken when interpreting the binding data.

Messenger RNA sequences corresponding to M_1, M_2, M_3, M_4, and M_5 receptors were all detected in HA and HV tissues, in agreement with the results from the binding assays. These experiments provide a second line of evidence for the presence of multiple subtypes of mAChR in the human heart. More conclusive data were acquired from the Western blot analyses of membrane proteins, which revealed the presence of proteins representing M_1, M_2, M_3, and M_5 receptors. Although M_4 was found to express at the mRNA level, the antibody targeting m_4 receptors failed to recognize M_4 proteins in human hearts, even when high concentrations of antibody and membrane proteins were used. This finding is unlikely to be due to a failure of the antibody used, because M_4 receptors were readily detected in rat brain preparations using the same antibody. Further studies are required to clarify whether the lack of M_4 protein expression is due to translational or post-translational mechanisms.

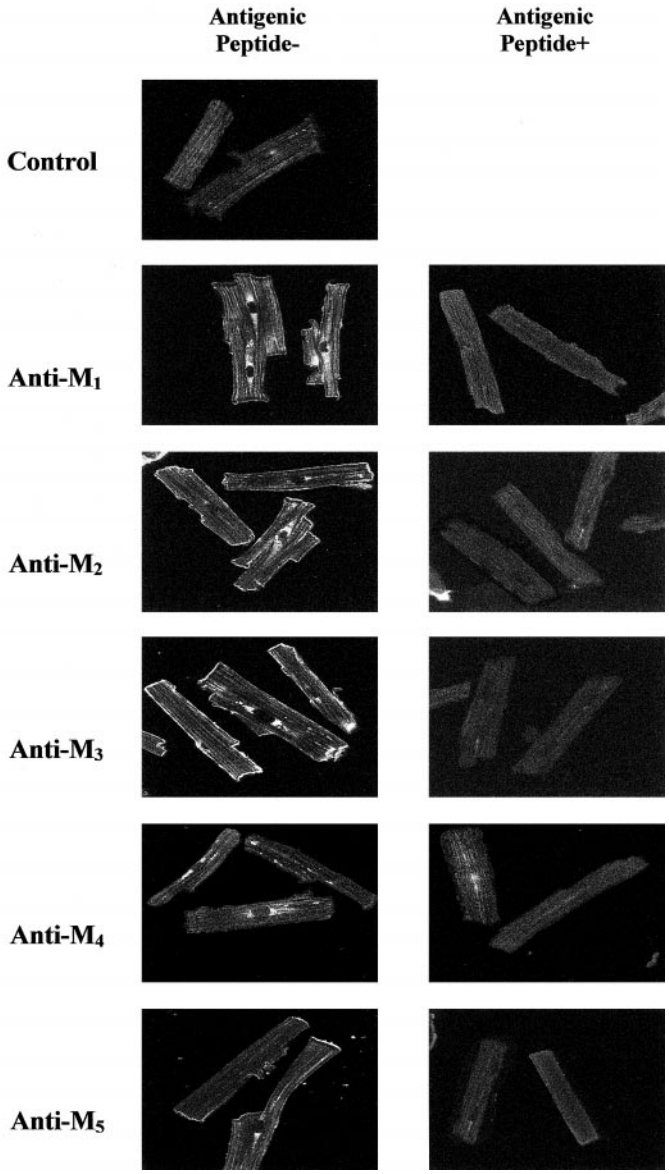

Fig. 4. Immunostaining of isolated human ventricular myocytes with antibodies directed against various mAChR subtypes. Except for anti-m_4 antibodies, antibodies against other subtypes of mAChR all show positive staining. Note that the anti-M_5 antibody stains only the intercalated discs and the anti-M_3 antibody preferentially stains the intercalated discs relative to other areas of the plasma membrane. Similar results were obtained from a total of three hearts.

In addition, our immunoblotting data also reveal a heterogeneous distribution of mAChR subtypes between HA and HV. We found that, whereas the M_1 and M_2 subtypes were expressed at equivalent levels in HA and HV, M_3 and M_5 receptors were distributed heterogeneously. M_3 receptors were about 10-fold sparser in HV versus HA, and in sharp contrast, the density of M_5 receptors was about 5-fold higher in HV than in HA.

The total density of mAChRs in HA was not significantly different from that in HV, as suggested by the similar maximum capacity [^{3}H]NMS binding capacity in each (Fig. 1; Table 2). We are unable at present to provide a quantitative comparison regarding the relative density of various subtypes of mAChR in HA or HV. Although the use of purified receptors or antigenic peptides can help to establish absolute quantities of various mAChR subtypes by generating standard curves on immunoblotting, adequate quantities of purified mAChRs are not currently available and the antigenic peptides available are too short to be detected.

Our control RT-PCR experiments (Fig. 3B) make it unlikely that any of the mAChR signals that we detected was due to contamination by noncardiac sources. This notion is supported by the immunohistochemical data, which were in full agreement with the immunoblotting results. Antibodies directed against M_1, M_2, M_3, and M_5 all produced clear positive staining of the plasma membrane, whereas the antibody to M_4 receptors failed to show any membrane staining.

Previous Studies Related to mAChR Subtypes in Hearts. Expression of multiple isoforms of mRNA encoding different subtypes of mAChRs (M_1/M_2/M_3/M_4) in chick hearts has been reported by two groups (Tietje and Nathanson, 1991; Gadbut and Galper, 1994). Sun et al. (1996) studied the antagonism of carbachol-induced chronotropy and inositol monophosphate accumulation in neonatal rat ventricular myocytes. They found that HHSiD, an M_3-selective antagonist, blocked carbachol effects, whereas pirenzepine and AF-DX 116 (M_1 and M_2 antagonists) had no effect. They speculated that neonatal ventricular myocytes have a heterogeneous population of muscarinic receptors including M_2 and M_3 subtypes. Sharma et al. (1996, 1997) used single-cell PCR, subtype-specific antibodies, and the measurement of Ca^{2+} transients to provide convincing molecular and functional evidence for the presence of M_1 receptors in rat ventricular myocytes. Ford et al. (1992) analyzed mAChR-mediated phosphoinositide hydrolysis in guinea pig atria and ventricles. The inhibition of the response to agonist by sev-

eral antagonists, including HHSiD and *p*-F-HHSiD, generated an affinity profile dissimilar to the pure M_2 response, suggesting "a second population of muscarinic sites". In the isolated rabbit heart, acetylcholine increased prostaglandin synthesis and the effects were inhibited by low concentration of 4-DAMP (10 nM) (Jaiswal et al., 1989). Although the investigators considered 4-DAMP an M_2 antagonist, the concentration they studied would probably block M_3 receptors, with minimal effects on M_2 receptors. The same group (Kan et al., 1996) has recently reevaluated the mAChR subtypes involved in prostacyclin synthesis and the authors now believe that acetylcholine can function via M_3 receptors in ventricular myocytes. In isolated blood-perfused dog atria, Akahane et al. (1990) compared the inhibitory potency of 4-DAMP, AF-DX 116, and pirenzepine for carbachol-induced negative chronotropic and inotropic responses. They found that the potency of 4-DAMP (an M_3-preferring antagonist) was equal to that of atropine but greater than AF-DX 116 (an M_2-preferring antagonist) and much greater than pirenzepine (an M_1-selelctive inhibitor), suggesting a role of the M_3 subtype. Yang et al. (1992) performed a detailed pharmacological characterization of mAChR subtypes in membrane homogenates from dog left ventricular tissues. Their data favored the existence of M_2 and M_3 subtypes, and argued against the presence of M_1 receptors. Because of the imperfect selectivity of available pharmacological probes, none of these studies provided clear and unequivocal definitions of mAChR subtype distribution.

To our knowledge, the present work is the first systematic study of mAChR subtype expression in human hearts. Previous studies found only M_2 receptors in human hearts, with the inability to reveal other subtypes due presumably to the lack of subtype-selective antagonists. For example, Giraldo et al. (1988) suggested, based on binding data, that there is a single subtype of mAChR (M_2) in HA and HV. However, they used only two subtype-selective antagonists, pirenzepine (M_1) and AF-DX 116 (for M_2).

Potential Significance. One of the major innovations in the field of the cholinergic nervous system was the discovery of multiple subclasses of muscarinic receptors. Although many cellular responses to mAChR stimulation are mediated by the various subtypes of mAChR, M_2 receptors are commonly believed to be the only functional mAChR subtype in the heart (Dörje et al., 1991; Gadbut and Galper, 1994; Mizushima et al., 1987; Pappano, 1991; Tietje and Nathanson, 1991). In many studies, the heart is often taken as a model for the exclusive presence of M_2 receptors. The present study, in conjunction with previous findings in other species, strongly suggests that multiple subtypes, and not just M_2 receptors, coexist in mammalian hearts. It seems that the one-receptor (M_2) concept needs to be revised and the potential participation of other mAChR subtypes in the cholinergic control of heart function must be considered. Prior work indicates that there are large species variations in terms of the subtypes of mAChR expressed in the hearts. M_1 and M_2 subtypes exist in rat hearts (Sharma et al., 1996). M_2, M_3, and M_4, but not M_1 and M_5, receptors are present in dog hearts (H. Shi, H. Wang, and Z. Wang, unpublished data; Shi et al., 1999a) and there are M_2, M_3, and M_5 receptors in guinea pig hearts (H. Shi, H. Wang, and Z. Wang, unpublished data; Shi et al., 1999b). Thus, extrapolation of data from animal species to humans must be done with caution.

Compared with other subtypes of mAChR, the M_5 receptor is probably the least known subtype in terms of its functional role in primary tissues, although its potential physiological function has been studied in heterologous expression systems (Kohn et al., 1996; Kukkonen et al., 1998; Reever et al., 1997). The present study provides the first evidence for the presence of an endogenous M_5 receptor subtype in a peripheral non-neuronal tissue (Reever et al., 1997). Although the function of the M_5 receptor is still unknown, our findings point to a potential functional role in the human heart.

We previously reported that stimulation of M_3 receptors by choline (Shi et al., 1999b), pilocarpine (Wang et al., 1999b), or tetramethylammonium (Shi et val., 1999a) in dog and guinea pig atrial myocytes activates a novel K^+ current with both delayed and inward rectifier properties. Activation of M_3 receptors caused significant slowing of the heart rate and shortening of the action potential duration. Stimulation of M_4 receptors by 4-aminopyridine also induces a distinct K^+ current in dog cells (Shi et al., 1999a). Sharma et al. (1996) found that activation of M_1 receptors stimulates Ca^{2+} transients in rat heart. The present study suggests potential relevance of the above-mentioned physiological observations to humans. Nevertheless, further direct experimentation is necessary to establish the function of M_1, M_3, and M_5 receptors in the human heart.

Our immunoblotting analysis indicated that M_3 and M_5 distributions are quantitatively different between HA and HV, with M_3 density higher in HA than in HV and M_5 higher in HV than in HA. In addition, immunostaining also revealed a characteristic localization of M_5 receptors to the intercalated discs. Other receptor subtypes were more evenly distributed throughout the surface membrane, although M_3 receptors also demonstrated a stronger staining on the intercalated discs relative to other regions of the plasma membrane. The data indicate that different subtypes of mAChR might have different patterns of cellular localization. Whether this heterogeneous distribution has functional implications awaits future studies.

In summary, we have obtained evidence with receptor binding, mRNA expression, and protein detection in support of the presence of multiple mAChR subtypes in human hearts. The tissue distribution (HV versus HA) and cellular localization vary among subtypes. These findings may open up opportunities for improved understanding of the subtype specificity of mAChR modulation of heart function and for the development of new drugs selectively targeting different subtypes of mAChRs.

Acknowledgments

We thank XiaoFan Yang for excellent technical assistance.

References

Akahane K, Furukawa Y, Karasawa Y and Chiba S (1990) Muscarinic receptor subtypes mediating negative chrono- and iontropic responses in isolated blood-perfused dog right atria. *J Auton Pharmacol* **10:**39–48.

Araujo D, Lapchak P and Quirion R (1991) Heterogeneous binding of [^{3}H]4-DAMP to muscarinic cholinergic sites in the rat brain: evidence from membrane binding and autoradiographic studies. *Synapse* **9:**165–176.

Barlow R and Shepherd M (1986) A further search for selective antagonists at M_2 muscarinic receptors. *Br J Pharmacol* **89:**837–843.

Bonner T, Buckley N, Young A and Brann M (1987) Identification of a family of muscarinic acetylcholine receptor genes. *Science (Wash DC)* **237:**237–240.

Bonner TI, Young AC, Brann MR and Buckley NJ (1988) Cloning and expression of the human and rat m_5 muscarinic acetylcholine receptor genes. *Neuron* **1:**403–410.

Brann MR, Ellis J, Jørgensen, Hill-Eubanks D and Penelope Jones SV (1993)

Muscarinic acetylcholine receptor subtypes: localization and structure/function, in *Progress in Brain Research* (Cuello AC ed) vol 98, pp 121–127, Elsevier Publishing Co, Amsterdam.
Dixon J and McKinnon D (1994) Quantitative analysis of potassium channel mRNA expression in atrial and ventricular muscle of rats. *Circ Res* **75:**252–260.
Dörje F, Wess J, Lambrecht G, Tacke R, Mutschler E and Brann M (1991) Antagonist binding profiles of five cloned human muscarinic receptor subtypes. *J Pharmacol Exp Ther* **256:**727–733.
Doods H, Mathy M, Davidesko D, Van Charldorp K, De Jonge A and Van Zwieten P (1987) Selectivity of muscarinic antagonists in radioligand and in vivo experiments for the putative M_1, M_2 and M_3-receptors. *J Pharmacol Exp Ther* **246:**929–934.
Eglen RM and Watson N (1996) Selective muscarinic receptor agonists and antagonists. *Pharmacol Toxicol* **78:**59–68.
Ford AP, Eglen RM and Whiting RL (1992) Analysis of muscarinic cholinoceptors mediating phosphoinositide hydrolysis in guinea pig cardiac muscle. *Eur J Pharmacol* **225:**105–112.
Gadbut AP and Galper JB (1994) A novel M_3 muscarinic acetylcholine receptor is expressed in chick atrium and ventricle. *J Biol Chem* **269:**25823–25829.
Giraldo E, Martos F, Gomez A, Garcia A, Vigano MA, Ladinsky H and Sanchez de la Cuesta (1988) Characterization of muscarinic receptor subtypes in human tissues. *Life Sci* **43:**1507–1515.
Goyal R (1989) Muscarinic receptor subtypes. Physiology and clinical implications. *N Engl J Med* **321:**1022–1028.
Hulme EC, Birdsall NJ and Buckley NJ (1990) Muscarinic receptor subtypes. *Annu Rev Pharmacol Toxicol* **30:**633–673.
Jaiswal N, Lambrecht G, Mutschler E and Malik KU (1989) Effect of M_2 muscarinic receptor antagonist 4-DAMP, on prostaglandin synthesis and mechanical function in the isolated rabbit heart. *Gen Pharmacol* **20:**497–502.
Kan H, Ruan Y and Malik KU (1996) Localization and characterization of the subtypes of muscarinic receptor involved in prostacyclin synthesis in rabbit heart. *J Pharmacol Exp Ther* **276:**934–941.
Kohn EC, Alessandro R, Probst J, Jacobs W, Brilley E and Felder CC (1996) Identification and molecular characterization of a m_5 muscarinic receptor in A2058 human melanoma cells. Coupling to inhibition of adenylyl cyclase and stimulation of phospholiase A2. *J Biol Chem* **271:**17476–17484.
Kukkonen JP, Nasman J, Rinken A, Dementjev A and Akerman KE (1998) Pseudononcompetitive antagonism of M_1, M_3 and M_5 muscarinic-mediated Ca^{2+} mobilization by muscrinic antagonists. *Biochem Biophys Res Commun* **243:**41–46.
Lazareno S and Birdsall N (1993) Pharmacological characterization of acetylcholine-stimulated [^{35}S]-GTP gamma S binding mediated by human muscarinic m_1-m_4 receptors: antagonist studies. *Br J Pharmacol* **109:**1120–1127.
Lazareno S, Buckley and Roberts F (1990) Characterization of muscarinic M_4 binding sites in rabbit lung, chicken heart, and NG108-15 cells. *Mol Pharmacol* **38:** 805–815.
Michel D, Stefanich E and Whiting R (1989) Direct labeling of rat M_3 muscarinic receptors by [^{3}H]4-DAMP. *Eur J Pharmacol* **166:**459–466.
Michel AD and Whiting RL (1988) Methoctramine, a polymethylene tetraamine, differentiates three subtypes of muscarinic receptor in direct binding studies. *Eur J Pharmacol* **145:**61–66.
Mitchelson F (1988) Muscarinic receptor differentiation. *Pharmacol Ther* **37:**357–423.
Mizushima A, Uchida S, Zhou X, Kagiya T and Yoshida H (1987) Cardiac M_2 receptors consist of two different types, both regulated by GTP. *Eur J Pharmacol* **135:**403–409.
Mutschler E, Moser U, Wess J and Lambrecht L (1989) Muscarinic subtypes: agonists and antagonists. *Prog Pharmacol* **7:**3–31.
Pappano AJ (1991) Vagal stimulation of the heartbeat: muscarinic receptor hypothesis. *J Cardiovasc Electrophysiol* **2:**262–273.
Peralta E, Ashkenazi A, Winslow J, Smith D, Ramachandran J and Capon D (1987) Distinct primary structures, ligand-binding properties and tissue-specific expression of four human muscarinic acetylcholine receptors. *EMBO J* **6:**3923–3929.
Reever CM, Ferrari-DiLeo G and Flynn DD (1997) The M_5 (m_5) receptor subtype: fact or fiction? *Life Sci* **60:**1105–1112.
Shi H, Wang H and Wang Z (1999a) Identification and characterization of multiple subtypes of muscarinic acetylcholine receptors and their physiological functions in canine hearts. *Mol Pharmacol* **55:**497–507.
Shi H, Wang H and Wang Z (1999b) Choline modulates cardiac membrane repolarization by activating an M_3 muscarinic receptor and its coupled K^+ channel. *J Membr Biol* **169:**55–64.
Sharma VK, Colecraft HM, Rubin LE and Sheu SS (1997) Does mammalian heart contain only the M_2 muscarinic receptor subtype? *Life Sci* **60:**1023–1029.
Sharma VK, Colecraft HM, Wang DX, Levey AI, Grigorenko EV, Yeh HH and Sheu SS (1996) Molecular and functional identification of m_1 muscarinic acetylcholine receptors in rat ventricular myocytes. *Circ Res* **79:**86–93.
Sun LS, Huber F, Robinson RB, Bilezikian JP, Steinberg SF and Vulliemoz Y (1996) Muscarinic receptor heterogeneity in neonatal rat ventricular myocytes in culture. *J Cardiovasc Pharmacol* **27:**455–461.
Tietje KM and Nathanson NM (1991) Embryonic chick heart expresses multiple muscarinic acetylcholine receptor subtypes. Isolation and characterization of a gene encoding a novel m_2 muscarinic acetylcholine receptor with high affinity for pirenzepine. *J Biol Chem* **266:**17382–17387.
Wang Z, Feng J, Shi H, Nerbonne JM and Nattel S (1999a) The potential molecular basis of different physiological properties of transient outward K^+ current in rabbit and human hearts. *Circ Res* **84:**551–561.
Wang H, Shi H and Wang Z (1999b) Pilocarpine modulates the cellular electrical properties of mammalian hearts by activating a cardiac M_3 receptor and a K^+ current. *Br J Pharmacol* **162:**1725–1734.
Wang Z, Yue L, White M, Pelletier G and Nattel S (1998) Differential expression of inward rectifier potassium channel mRNA in human atrium versus ventricle and in normal versus failing. *Circulation* **98:**2422–2428.
Watson M, Yamamura H and Roeske W (1983) A unique regulatory profile and regional distribution of [^{3}H]pirenzepine binding in the rat provide evidence for distinct m_1 and m_2 muscarinic receptor subtypes. *Life Sci* **32:**3001–3010.
Wotta DR, Wattenberg EV, Langason RB and el-Dakahany EE (1998) M_1, M_3 and M_5 muscarinic receptors stimulate mitogen-activated protein kinase. *Pharmacology* **56:**175–186.
Yang CM, Yeh HM, Sung TC, Chen FF and Wang YY (1992) Characterization of muscarinic receptor subtypes in canine left ventricular membranes. *J Recept Res* **12:**427–449.
Yue L, Melnyk P, Gaspo R, Wang Z and Nattel S (1999) The molecular mechanism of ionic remodelling of repolarization in a dog model of atrial fibrillation. *Circ Res* **84:**776–784.
van Zwieten PA and Doods HN (1995) Muscarinic receptor and drugs in cardiovascular medicine. *Cardiovasc Drugs Ther* **9:**159–167.

Send reprint requests to: Zhiguo Wang, Ph.D, Research Center, Montreal Heart Institute, 5000 Bélanger St. East, Montreal, Quebec, Canada H1T 1C8. E-mail: wangz@icm.umontreal.ca