Pharmacological Comparison of the Cloned Human and Rat M2 Muscarinic Receptor Genes Expressed in the Murine Fibroblast (B82) Cell Line

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ABSTRACT

The coding sequence of the human m2 receptor gene was amplified by polymerase chain reaction and stably transfected into a murine fibroblast cell line (B82). We have compared the human M2 clonal cell line (HM2-B10) with the previously established B82 cell line (M2LKB2–2) expressing the rat M2 receptor to assess drug specificity, drug selectivity and effector coupling. Both transfected cell lines showed a high level of specific, saturable [3H](−)-N-methyl-3-quinuclidinyl benzilate binding with Kd values of 243 pM (155–352 pM) and 345 pM (234–539 pM) and Bmax values of 97 ± 4 and 338 ± 16 fmol/10^6 cells, respectively. Inhibition of [3H](−)-N-methyl-3-quinuclidinyl benzilate binding to HM2-B10 cells and M2LKB2–2 cells showed the same rank order of potency for the antagonists: atropine > dextemetide > 4-diphenylacetatoxy-N-methylpiperidine methiodide > himbacine > methoctramine > 11-[2-[(diethylamino)ethyl]-1-piperidinyl]acetyl]-5,11-dihydro-6H-pyrido-[2,3-b][1,4]benzodiazepine-6-one > hexahydro-sila-difenidol hydrochloride > pirenzepine. Correlation analysis of the pKi values indicate that the expressed human and rat M2 receptors have nearly identical ligand-binding characteristics. Carbachol inhibited forskolin-stimulated cAMP formation with similar potencies in both cell lines [EC_{50} = 2.4 μM (0.2–2.8) and 1.1 μM (0.2–5.3) for the human and rat M2 receptor, respectively]. In the M2LKB2–2 cells, carbachol slightly stimulated the [3H]inositol monophosphate formation but had no significant effect in HM2-B10 cells. In conclusion, the human and rat M2 receptors expressed in the B82 cell line have very similar binding properties but exhibit slight differences in effector coupling mechanisms.

mACHRs have been classified pharmacologically into three subtypes (M1, M2 and M3) according to their affinities for different antagonists (Hammer et al., 1980). Conventional pharmacological studies on muscarinic receptors have been difficult because of the lack of subtype-specific ligands and the fact that different tissues express a mixture of different subtypes. The muscarinic receptors of the heart belong predominantly to the M2 subtype, although a small population of M1 sites also are present (Sharma et al., 1997; Watson et al., 1983). The M2 receptor has very important regulatory control over heart rate (negative chronotropic) and myocardial contractility (negative inotropic). Many cardiac and non-cardiac drugs bind to these receptors, causing serious side effects. A number of models have been used to study these cardiac muscarinic receptors. Primary cultures of myocytes from adult or fetal rat heart are hard to maintain and are often contaminated with other cell types. Furthermore, myocyte cultures are obtained from animal (mostly rat) tissues, although ultimately, pharmacological data must be obtained for drugs used in humans.

Molecular biological techniques have made possible the separate characterization of a single receptor population in transfected mammalian cells expressing a high density of a particular receptor subtype. Five muscarinic receptor genes (m1–m5) (Bonner et al., 1987, 1988; Kubo et al., 1986a, 1986b; Peralta et al., 1987a, 1987b) encoding highly related receptor proteins have been cloned from different species. The human (Peralta et al., 1987a) and the rat (Lai et al., 1990) m2 receptor show 100% amino acid homology in the putative transmembrane and extracellular loop regions. Site-specific molecular modeling suggests that the human m2 receptor protein would have a helical structure with a putative transmembrane and extracellular loop regions. The human m2 receptor contains a proline-rich region in the N-terminus, which is thought to be involved in receptor oligomerization.
directed mutagenesis and affinity labeling experiments implicate these regions in the ligand binding; therefore, the binding characteristics of the rat and human M2 receptors are expected to be very similar. However, the results available in the literature for the cloned M2 receptors indicate species differences for the affinity of AF-IX 116 (11-[(diethylamino)methyl]-1-piperidinyl)acetyl)-5,11-dihydro-6H-pyrido[2,3-b](1,4)-benzodiazepine-6-one and PZ (Akiba et al., 1988; Buckley et al., 1989; Lai et al., 1990).

The cloned M2 receptor is known to be inversely coupled to cAMP formation (Peralta et al., 1988) and has some stimulatory effect on IP3 formation. The differences between the rat and the human M2 receptors are found almost exclusively in the putative third intracellular loop region (fig. 1). The third intracellular loop is thought to have an important role in determining the specificity of the G protein coupling. This suggests that there can be some differences in the second-messenger coupling between the human and rat M2 receptors.

The rat m2 gene expressed in vitro in mammalian cells showed the characteristics of the pharmacologically defined M2 receptors (Lai et al., 1990). In our study, we report the cloning and expression of the human M2 receptor in the B82 fibroblast cell line. To examine the possible species differences, we compared the binding properties and effector coupling of the human M2 receptor with those of the rat M2 receptor, both expressed in the same B82 cell line using whole-cell preparations and physiological conditions. In addition, we performed radioligand binding assays on the human clone with several nonmuscarinic compounds (including nicotinic, cardiac and neuroleptic drugs) to examine possible drug interactions.

Methods

Amplification of the coding sequence of the human muscarinic m2 receptor gene by PCR. The open reading frame of the human m2 receptor gene was amplified from human placental genomic DNA (Clontech, Palo Alto, CA) by PCR (Saiki et al., 1985).

Two specific primers based on the human cardiac m2 receptor sequence (Peralta et al., 1987a) were synthesized using a Cyclone Plus DNA synthesizer (Milligen Bio-Research, Wavago, CA). The primers were purified by anion exchange chromatography, using OLIGO-PACK columns (Milligen/Biosearch, Burlington, MA).

The 5′ primer (5′-ACCTCCACATGAAACTCAAAACTCC-3′) carried a SalI restriction site (underlined) as a linker. The 3′ primer included a HindIII restriction site linker sequence (underlined): 5′-CGAAGCTTTCATCTTGGCGCC-TATGTT-3′. The PCR amplification was carried out in a Programmable Cyclic Reactor (ericomp, San Diego, CA). In the reaction, we used Taq DNA polymerase and Gene Amp DNA amplification kit (Perkin-Elmer Cetus, Norwalk, CT). The reaction mixture contained 200 μM concentration of each dNTP, 1 μg of each primer and 1 μg of the human genomic DNA. The conditions were the following: initial denaturation at 94°C for 2 min, annealing at 55°C for 20 sec, chain extension at 72°C for 1 min, denaturation at 94°C for 20 sec (25 cycles) and a final extension step of 10 min at 72°C. The PCR product was analyzed on 0.8% agarose gel and isolated from the gel by electrophoresis.

Subcloning and sequencing. The purified PCR product was subcloned into pGEM3Zf−) vector (Promega, Madison, WI) with homopolymer tailing method (Boehringer-Mannheim Biochemical, Mannheim, Germany) using poly(G)-tail on the PCR product and poly(C)-tail on the vector. The sequence of the subcloned DNA was determined by the dideoxy-chain termination method (Sanger et al., 1977) using Sequenase Version 2.0 sequencing kit (United States Biochemical, Cleveland, OH) on 5% polyacylamide gel prior to autoradiography. The sequence data were analyzed with the Geneius DNA analysis program (Hoeffer Scientific, San Francisco, CA) and compared with the published hm2 sequence showed 100% homology.

In vitro expression of the human m2 muscarinic receptor gene in murine fibroblast (B82) cell line. The cloned and sequenced DNA was excised from the recombinant pGEM3Zf−) with SalI and HindIII restriction enzymes. The fragment was ligated into the expression vector pHAPl-1-neo (a gift from Dr. L. Kedes, Stanford University, Stanford, CA), which has a SalI/HindIII cloning site downstream from the human β-actin promoter (Gunning et al., 1987).

The recombinant plasmid (10 μg) was transfected into the murine fibroblast (B82) cells using the calcium phosphate precipitation method (Graham and Van der Eb, 1973). The transfected cells were selected for the expression of neomycin resistance with 500 μg/ml geneticin (G418; Gibco BRL, Grand Island, NY). Clones with stable, high-affinity [3H]−MQN binding were maintained in a nonselecting medium (5% fetal calf serum, 5% newborn calf serum, 45% Ham’s F-12, 45% Dulbecco’s modified Eagle’s medium, 100 U/ml penicillin and 100 μg/ml streptomycin) in a humidified atmosphere with 95% air/5% CO2.

All assays were carried out on intact cells; 50,000 cells/well were plated out on 24-well tissue plates (Costar, Cambridge, MA) 48 hr before each assay. The cells were counted on the day of the assay. G418 was omitted throughout these experiments.

Radioligand binding assays. [3H]−MQN binding (87.4 Ci/mmol; New England Nuclear, Boston, MA) binding assay on the intact

Fig. 1. Comparison of the deduced amino acid sequences of the rat (Lai et al., 1990) and the human M2 receptors. The putative transmembrane domains are underlined. *Amino acid differences. Boldface letters indicate the differences between the sequences published by Gocayne et al. (1987) and Lai et al. (1990).
transfected B22 cells was carried out as described previously (Mei et al., 1989b). The saturation assays were performed using 32.5 to 951 pM $[^{3}H]$-MQNB. Ligand/$[^{3}H]$-MQNB competition assays were carried out using 10 concentrations of the competing ligands and an average concentration of 341 pM $[^{3}H]$-MQNB. The ligands used include atropine sulfate, carbachol, quinidine (all from Sigma Chemical, St. Louis, MO); AF-DX 116, PZ (both were gifts from Dr. K. Thomae, Biberach, Germany); 4-DAMP, methoctramine, d-tubocurarine, hemicholinium-3, gallamine, HHSID, dextetimide, levetimide (from RBI, Natick MA); himbacine (gift from Dr. W. Taylor); and procainamide (gift from Squibb Institute) Both saturation and competition assays were conducted in 1 ml of IMDM at 37°C for 3 hr. Specific binding was determined as the amount of binding inhibited by 1 μM atropine sulfate. Radioactivity was measured by liquid scintillation counting (Searle Analytic 81; 45% efficiency.)

**cAMP formation assay.** The cAMP formation studies were performed according to a modified method of Gilman (1970). The cAMP formation was stimulated with 100 μM forskolin ([β-deacetyl-β-y-N-methylpiperazino]-butyryl dihydrochloride) (Cal-Biochem, La Jolla, CA) in the presence of 5 mM IBMX (Sigma, St. Louis, MO) in IMDM for 3 min at 37°C, with or without 10 pM to 10 mM carbachol or 1 μM atropine. The preparation of the cell extracts and the measurement of the cAMP content were carried out as described previously (Mei et al., 1989a).

**Inositol lipid hydrolysis studies.** A method modified from that described by Berridge et al. (1982) was used to measure the accumulation of $[^{3}H]$IP$_1$ after the stimulation by 100 pM to 10 mM carbachol with or without 1 μM atropine in the presence of 10 mM lithium chloride. The experimental procedure was essentially described previously (Mei et al., 1989b) with 5 mM sodium tetraborate/60 mM sodium formate instead of myoinositol during ion exchange chromatography before eluting the $[^{3}H]$IP$_1$, with 2 ml of 0.2 M ammonium formate/0.1 M formic acid. The radioactivity in the eluate was determined by liquid scintillation spectrophotometry.

**Data analysis.** The ligand binding data were analyzed by logistic nonlinear least-squares analysis using a computerized iterative procedure developed by S. Yamamura for the Apple computer. In the inhibition experiments, the IC$_{50}$ values were corrected to Kd values using the Cheng-Prusoff equation (1973), results are presented as the geometric mean (range).

Statistical differences were analyzed using the one-way ANOVA for grouped data followed by paired t test. A value of P < .05 was considered statistically significant.

**Results**

The PCR using the two gene-specific primers for the human M2 muscarinic receptor yielded the selective amplification of a 1.4-kb DNA fragment from human genomic DNA. The fragment was subcloned into pGEM3Zf(+) vector and sequenced. The sequence analysis showed 100% homology with the published sequence.

**In vitro expression and characterization of the amplified human m2 muscarinic receptor gene in the murine fibroblast (B22) cell line.** The untransfected B22 cells did not exhibit any specific $[^{3}H]$-MQNB binding. After the transfection, 26 clones selected by G418 resistance showed high-level specific binding for $[^{3}H]$-MQNB. One of the clones, HM2-B10, which had the highest specific binding for $[^{3}H]$-MQNB, was chosen for the pharmacological characterization. In parallel with each experiment on the human HM2-B10 cell line, we did the pharmacological characterization on the rat M2LKB2–2 cells (Lai et al., 1990).

**Pharmacological characterization.** The G418-resistant clonal cell line HM2-B10 showed a high level of specific, saturable $[^{3}H]$-MQNB binding (fig. 2A) with a Kd value of 345 pM (234–539 pM) and a pseudo-Hill coefficient of 1.8 (fig. 2B). The rank order of potency of these antagonists in inhibiting $[^{3}H]$-MQNB binding was atropine > himbacine > methoctramine > AF-DX 116 > PZ. Carbachol inhibited $[^{3}H]$-MQNB binding on the HM2-B10 cells with a Kd value of 1.8 μM (1.7–1.9 μM) and a pseudo-Hill coefficient of 1.1 ± 0.33. These results are characteristic of the pharmacologically defined M2 type receptor that predominates in cardiac tissue.
The assay conditions are described in the text. The IC_{50} values and Hill coefficients are summarized in Table 1. Inhibition of [3H]-MQNB binding by 13 muscarinic drugs. The inhibition constants (K_i) values and pseudo-Hill coefficients of the respective ligand/[3H]-MQNB competition isotherms for the human and rat M2 cells are summarized in table 1. To compare the affinities of these 13 drugs at the human and rat M2 receptors, a correlation analysis was performed by plotting the negative logarithm of the K_i value (pK_i) of the HM2-B10 (human M2) cells against the pK_i value of the M2LKB2–2 (rat M2) cells (fig. 4). The result showed a very high correlation (r = .998); the slope of the correlation line was close to unity (0.98).

We previously tested the potencies of several nonmuscarinic drugs used in the treatment of cardiovascular disease conditions to interact with the cardiac muscarinic receptors by measuring the inhibition of [3H]QNB binding to rabbit heart homogenates (Fields et al., 1978). In the present study, we tested the ability of the same 52 nonmuscarinic drugs to compete for [3H](–)MQNB binding sites on the human HM2-B10 cells under physiological buffer conditions. The K_i values and pseudo-Hill coefficients for the most potent drugs are shown in table 2. The nicotinic drugs decamethonium and d-tubocurarine as well as the choline reuptake inhibitor hemicholinium-3 had potency in inhibiting [3H](–)MQNB binding with K_i values in micromolar concentrations. The commonly used antiarrhythmic agents quinidine and propranolol, the K_i value in the low micromolar range. The tricyclic antidepressant imipramine had a K_i value of 0.13 μM, which may account for its cardiotoxicity. Other compounds that were able to inhibit [3H](–)MQNB by 40% to 50% concentrations of 100 μM include the beta adrenergic blockers labetalol and metoprolol, the beta adrenergic agonist dobutamine, the K_i–sparing diuretics spironolactone and amiloride, the cholesterol synthesis inhibitor lovastatin and the phosphodiesterase inhibitor papaverine. The ability of glycerin to inhibit [3H](–)MQNB binding by 68% even at 10 μM is probably due to nonspecific membrane perturbations.

**Functional coupling of the expressed mAChRs to cAMP formation.** Forskolin elevated the cellular cAMP level in a dose-dependent manner in both the transfected and nontransfected B82 cells. Forskolin (100 μM) elicited a maximal stimulation of cAMP formation that was 10-fold over the basal level. In the nontransfected B82 cells, carbachol had no effect on forskolin-stimulated cAMP formation (Mei et al., 1989a).

Neither carbachol at 10 μM nor atropine at 1 μM showed any effect on the basal cAMP formation in B82 cells transfected with the human m2 gene (HM2-B10). Carbachol (1 μM) elicited the maximal stimulation of cAMP formation that was 10-fold over the basal level in the nontransfected B82 cells. Forskolin elevated the cellular cAMP level in a dose-dependent manner in both the transfected and nontransfected B82 cells.

### Table 1

<table>
<thead>
<tr>
<th>Drug</th>
<th>HM2-B10 cells (human)</th>
<th>M2LKB2-2 cells (rat)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>K_i [nM]</td>
<td>n_H</td>
</tr>
<tr>
<td>Agonist</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Oxotremine</td>
<td>235 (214-254)</td>
<td>1.4 ± 0.5</td>
</tr>
<tr>
<td>2. Acetylcholine</td>
<td>347 (318-399)</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>3. Carbachol</td>
<td>1820 (1740-1930)</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>Antagonist</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Atropine</td>
<td>0.63 (0.5-0.7)</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>5. Dextemizine</td>
<td>1.2 (0.8-1.8)</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>6. 4-DAMP</td>
<td>4.7 (4.1-5.6)</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>7. Himbacine</td>
<td>12 (9.2-17)</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>8. Methoctramine</td>
<td>42 (38-49)</td>
<td>1.5 ± 0.4</td>
</tr>
<tr>
<td>9. AF-DX 116</td>
<td>67 (54-80)</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>10. HHSID</td>
<td>115 (110-190)</td>
<td>1.1 ± 0.3</td>
</tr>
<tr>
<td>11. Pirenzepine</td>
<td>374 (340-400)</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>12. Gallamine</td>
<td>1730 (1200-2080)</td>
<td>0.7 ± 0.2</td>
</tr>
<tr>
<td>13. Levetimide</td>
<td>9060 (4640-13,440)</td>
<td>0.9 ± 0.3</td>
</tr>
</tbody>
</table>

The intact cells were incubated with [3H](–)MQNB (final concentrations, 0.29-0.43 nM) and 10 concentrations of selected agonists or antagonists. IC_{50} values and Hill coefficients (n_H) are analyzed using a nonlinear least-squares regression analysis based on three separate experiments done in duplicate. Values are expressed as the geometric mean with the range in parentheses for K_i and arithmetic mean ± S.E.M. for the Hill coefficient.
pM to 10 mM) inhibited forskolin-stimulated cAMP formation in the HM2-B10 cells in a dose-dependent manner (F = 12.9, P < .01) (fig. 5A). The EC$_{50}$ value of carbachol was 2.4 μM (0.2–2.8 μM). Carbachol at 10 μM inhibited forskolin-stimulated cAMP formation by 54%.

In B82 cells transfected with the rat m2 homolog (M2LKB2–2), both the basal and forskolin (100 μM)-stimulated cAMP level was lower than that in the HM2-B10 cells, but the stimulation was still 10-fold over the basal level. Carbachol (10 pM to 10 mM) inhibited the cAMP formation in a dose-dependent manner (fig. 5B). The EC$_{50}$ value for carbachol was 1.1 μM (0.2–5.3 μM) (F = 29, P < .01). The forskolin-stimulated cAMP formation was suppressed by 70% at the 10 μM carbachol dose.

The difference between the two cell lines in inhibiting the cAMP formation is significant (F = 2.19, P < .05). The inhibitory effect of carbachol on forskolin-stimulated cAMP formation was antagonized by atropine in both cell lines. Atropine at 1 μM shifted the EC$_{50}$ value of carbachol to 250 μM (73–920 μM) in the HM2-B10 cells and 240 μM (60–660 μM) in the M2LKB2–2 cells (data not shown).

**Effect of carbachol on the hydrolysis of inositol lipids in the transfected cells.** In the nontransfected B82 cells, 100 μM carbachol has no effect on the basal [3H]IP$_1$ accumulation (Mei et al., 1989b). The basal [3H]IP$_1$ accumulation was the same in the HM2-B10 and M2LKB2–2 cells. Carbachol at 100 pM to 10 mM had no significant stimulatory effect in the human M2 cells (F = .6, P = NS, fig. 6A). In the rat M2 cells, the agonist carbachol significantly (t = 2.5, P < .05) stimulated the [3H]IP$_1$ accumulation at 10 μM concentration with an $E_{\text{max}}$ value of 1.8 ± 0.24-fold over the basal level. With the increase of the carbachol concentration, the level of the [3H]IP$_1$ accumulation declined. This response to the agonist was totally abolished by the addition of 1 μM atropine.

**Discussion**

The human m2 receptor gene was amplified by PCR and expressed in the murine fibroblast B82 cell line, which pre-
Fig. 6. The effect of carbachol and atropine (1 μM) on the stimulation of the [3H]IP₃ formation in (A) HM2-B10 cells and (B) M2LKB2–2 cells. The data points represent the mean ± S.E.M. of three determinations, expressed as ratios of total [3H]IP₃ accumulated/basal [3H]IP₃ accumulated obtained from either carbachol-treated (●) or carbachol plus atropine-treated (■) cells. The effect of carbachol in stimulating the [3H]IP₃ accumulation was not significant in the HM2-B10 cells (F = .6, P = NS). The Eₘₐₓ values for carbachol in stimulating [3H]IP₃ formation in M2LKB2–2 cells were 1.5 ± .2-fold over the basal level.

Previously lacked mACHRs. Binding and functional comparisons in parallel experiments were carried out with the previously cloned rat m2 receptor gene expressed in the B82 cells. The results of these studies are (1) the human m2 gene was successfully expressed in the B82 cells, (2) the human and rat m2 receptors bound [3H](−)-MQNB with similar Kᵦ values, (3) the pKᵦ comparison showed high similarity between the rat and human clones for 13 muscarinic drugs, (4) the human m2 receptor genes bound [3H](−)-MQNB with similar Kᵦ values, and nucleotide concentrations, and therefore affinity values measured under these conditions will determine the occupancy of the receptors. For this reason in the binding experiments, more pharmacological data are needed from recombinant cell lines.

By cloning the human muscarinic m2 receptor gene, we were able to compare the human and rat muscarinic M2 receptors expressed in the same B82 cell line. The deduced amino acid sequence comparison between the human and the rat m2 receptor genes showed 96% homology. This is higher than expected because the rat m2 gene cloned in our laboratory (Lai et al., 1990) is slightly different from that previously published (Gocayne et al., 1987). Based on the amino acid sequence identity between the two genes in the transmembrane domains, we expected very similar binding properties. The differences between the two receptor proteins are located almost exclusively in the third intracellular loop. The regions of the third intracellular loop adjacent to the transmembrane domains are known to have a role in the coupling of the receptors to G proteins (Lechleiter et al., 1990; Wess et al. 1990, 1997). These regions are completely homologous between the human and rat receptors, whereas the middle section of the loop contains a number of substitutions. It is not known what effect these substitutions have on the secondary structure of the loop and, therefore, on the G protein coupling specificity. On the other hand, ligand binding and G protein coupling are allosteric processes, so it is conceivable that the differences in the conformation of the intracellular loop might have an allosteric effect on the ligand binding.

To compare the ligand binding and second-messenger coupling characteristics of the rat and human M2 receptors, the open reading frame of each gene was cloned from genomic DNA by PCR (the muscarinic receptor genes have no introns) and transfected by the same method into the same cell lines using the same expression vector. It is well known that the ligand affinity of a ligand to the receptor is modified by both intracellular and extracellular conditions. The relationship between the high- and low-affinity states in the signal transduction process is not established; however, it is reasonable to assume that the initial encounter between the ligand and the receptor in living tissue happens under physiological ion and nucleotide concentrations, and therefore affinity values measured under these conditions will determine the occupancy of the receptors. For this reason in the binding experiments, we used whole-cell preparations, and the assays were conducted in IMDM medium (1.5 mM Ca²⁺, 4.8 mM K⁺, 0.8 mM Mg²⁺ and 115 mM Na⁺) at 37°C.

The transfected cells exhibited stable, specific, high-affinity [3H](−)MQNB binding. The Kᵦ values were approximately the same in the human and rat cell clonal line. The Bₘₐₓ value of the human HM2-B10 cells was 3.5 times lower than the Bₘₐₓ value of the rat M2LKB2–2 cells (96.9 ± 3.8 and 338 ± 16 fmol/10⁶ cells, respectively). These values correspond to 510 fmol/mg protein for the HM2-B10 cells and 1778 fmol/mg protein for the M2LKB2–2 cells. The density of muscarinic receptors in heart tissue homogenates shows a

nisms of the drug action. Accumulating results from using different cell lines for the expression of the same receptor called our attention to the tissue specificity of the drug response. It is becoming increasingly clear that the cellular environment (G protein and effector pool) and the density of the expressed receptors can modify the response to the same drug even when it acts through the same receptor. To understand the complexity of the action of a drug in a living organism and to be able to select a valid model for drug screening, more pharmacological data are needed from recombinant cell lines.

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3–4-fold difference for different species (Fields et al., 1978). The maximal number of $[^3H](\cdot)$-QNB binding sites is 57 fmol/mg tissue in rabbit heart, 144 fmol/mg tissue in rat heart and 178 fmol/mg tissue in guinea pig heart. We also determined previously the regional distribution of $[^3H](\cdot)$-QNB binding sites in rat (Yamada et al., 1980), rabbit (Fields et al., 1978) and human (Roeske and Yamamura, 1980) heart. The atrial tissue generally contains six to nine times more muscarinic receptors (300 fmol/mg protein in rabbit left atrium) than ventricular tissue (53 fmol/mg protein in rabbit right ventricle).

The inhibition of the $[^3H](\cdot)$-MQNB binding by the muscarinic antagonists showed a rank order of potency typical for the cardiac muscarinic M2 receptors of atropine > hemicholine > methoctramine > AF-DX 116 > PZ > carbachol. Hemicholine (Wang et al., 1988), AF-DX 116 (Watson et al., 1986) and methoctramine (Melchiorre et al., 1987) have been shown to be selective for the M2 receptor. These ligands had much higher affinity for the rat and human M2 receptors than PZ, which has been shown to have low affinity for the M2-type receptor. Methoctramine showed a high pseudo-Hill coefficient in both cell lines, as has been found in neuronal and cardiac membranes (Giraldo et al., 1988; Melchiorre et al., 1987). This effect is probably due to an allosteric interaction of methochamine with the receptors. Carbachol inhibits $[^3H](\cdot)$-MQNB binding in the human and rat M2 cells with similar $K_i$ values (1.8 and 2 $\mu$M, respectively). The pseudo-Hill coefficient indicates a single-affinity state for this agonist in both cell lines. In rat cardiac tissue, there are two agonist affinity states of the M2 receptors resulting from interaction with G proteins (Watson et al., 1986). In CHO cells transfected with the porcine m2 receptor gene (Ashkenazi et al., 1987) and in the human kidney cells transfected with the human m2 receptor gene (Peralta et al., 1987a), multiple-affinity states were also observed for carbachol. This difference may be due to the different assay conditions. The $pK_a$ values for 10 muscarinic antagonist and 3 agonist drugs were highly similar between the human and rat clones with similar pseudo-Hill coefficient values.

In testing the human cell line with nonmuscarinic compounds, we found that the classic nicotinic cholinergic drugs d-tubocurarine and decamethonium showed potency in inhibiting $[^3H](\cdot)$-MQNB binding. These compounds at high concentrations appear to interact with both muscarinic and nicotinic receptors, as was demonstrated in rabbit heart tissue (Fields et al., 1978). Hemicholinium-3, a choline uptake inhibitor, has been shown to effect the muscarinic receptors in rabbit heart (Fields et al., 1978), and we could also see an effect in the HM2-B10 cells. Imipramine, a tricyclic antidepressant with severe cardiotoxicity (Spiker et al., 1976), had affinity for the human M2 receptors at concentrations below the clinically used level. The action on the muscarinic receptors may play a role in this serious side effect. The antiarrhythmic, cardioactive drugs quinidine and procainamide were also tested on the HM2-B10 cells. Quinidine had higher potency in inhibiting the $[^3H](\cdot)$-MQNB binding than procainamide, but both exhibited interactions with the muscarinic receptors. Among the neuroleptic drugs, the very potent and widely used chlorpromazine showed high affinity (215 nM) to the HM2-B10 cells, similar to the hm2-transfected CHO cells (Bolden et al., 1992). The marked anticholinergic side effects of this drug can be explained with its interactions with M2 muscarinic receptors.

To determine the interaction of the expressed M2 receptors with G proteins, we measured the effects of carbachol on two second-messenger systems: adenyl cyclase and phospholipase C. In the human M2 cell line, 100 $\mu$M forskolin stimulated the cAMP formation 10 times over the basal level. We could see the same effect on the rat M2 clone, but interestingly there was a difference in both the basal and forskolin-stimulated cAMP levels between the two cell lines. The human clone showed significantly higher basal and stimulated cAMP levels. Carbachol inhibited the cAMP formation in both cell lines, but there was a greater level of inhibition in the cells expressing the rat m2 cDNA. Carbachol (10 $\mu$M) inhibited forskolin-stimulated cAMP formation by 54% in the HM2-B10 (human M2) and 70% in the M2LKB2–2 (rat M2) cells. It was shown with other m2-transfected cells (Peralta et al., 1987a) that the increase in the $B_{max}$ has only a weak effect on the inhibition of cAMP formation. One possibility in our case is that in the transfected cells, the cAMP homeostasis was slightly altered because we saw differences in both the basal and maximal forskolin-stimulated cAMP levels before carbachol application. It was shown (Jakubik et al., 1995) that the mAChRs exhibit constitutive activity at high expression levels. This agonist-independent constitutive inhibition of adenyl cyclase at high receptor densities may explain the lower basal and forskolin-stimulated cAMP formation in the M2LKB2–2 (rat M2) cells.

The effect on the $[^3H]IP_1$ accumulation in the two cell lines was also different. In the human M2 cell line, carbachol had no significant effect on the $[^3H]IP_1$ accumulation, whereas in the cells expressing the rat M2 receptor, we could see a mild but significant stimulation of the inositol lipid level. This difference can be explained by the different $B_{max}$ value because it was shown with porcine M2 receptor (Ashkenazi et al., 1987) that the PI response is dependent on a higher level of receptor expression than the cAMP response. The physiological significance of this coupling is not clear. It is interesting to note that in the B82 cell system, the EC$_{50}$ values of carbachol in inhibiting the forskolin-stimulated cAMP formation and in stimulation of PI hydrolysis are approximately the same. Evidence now shows (Camps et al., 1992) that the pertussis toxin-sensitive stimulation of PI hydrolysis found for many G$_i$-coupled receptors is mediated by G protein beta gamma subunits through direct interaction with phospholipase C$\beta$2 or C$\beta$3. This response requires both high receptor expression level and high agonist concentrations. However, there also is evidence for a pertussis toxin-insensitive mechanism, which can act synergistically with the beta gamma stimulation (Zhu and Birnbaumer, 1996). On the other hand, the maximal stimulation of the PI hydrolysis was only 1.8-fold. This is much lower than was seen in the CHO cells expressing the porcine M2 receptors (Ashkenazi et al., 1987) and with human kidney cells expressing the human M2 receptor (Peralta et al., 1988) at similar receptor densities. It is possible that in B82 cells, one or more components of the above signaling mechanisms are not present.

Recombinant cell lines are very useful in the primary screening of candidate drugs and in the study of the signal transduction mechanisms initiated by a single receptor subtype without the interference of other subtypes. These systems generally have higher expression levels of the trans-
fected receptor population and can amplify some weak responses. These responses may not be noticed in tissue preparations in which an average response is measured but may be physiologically important for small subpopulations of cells with high receptor expression levels. The choice of the host cell line is very important because the second-messenger interaction with the expressed receptors depends on the coupling system of the host cells. To obtain reliable comparisons between subtypes or species, the same host cell lines with similar receptor densities should be used. To select an adequate model system, further work is needed to characterize the signal transduction machinery (G protein subunits, adenyl cyclase types, and so on) present in the host cells.

In conclusion, our results demonstrate that the human and rat M2 receptors are very similar to each other in binding characteristics and that the binding results correlate with the functional physiological data from heart tissue. However, there is a slight difference in the coupling characteristics of the two clones. Further experiments are necessary to determine whether the stimulation of PI hydrolysis found in the cells expressing the rat M2 receptors have physiological consequences in heart areas (e.g., left atria) with high M2 receptor densities.

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References


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