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ORIGINAL ARTICLE

The detection of the non-M_2 muscarinic receptor subtype in the rat heart atria and ventricles

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Abstract Mammal heart tissue has long been assumed to be the exclusive domain of the M_2 subtype of muscarinic receptor, but data supporting the presence of other subtypes also exist. We have tested the hypothesis that muscarinic receptors other than the M_2 subtype are present in the heart as minor populations. We used several approaches: a set of competition binding experiments with pirenzepine, AFDX-116, 4-DAMP, PD 102807, p-F-HHSiD, AQ-RA 741, DAU 5884, methoctramine and tripinamide, blockage of M_1 muscarinic receptors using MT7 toxin, subtype-specific immunoprecipitation experiments and determination of phospholipase C activity. We also attempted to block M_1–M_4 receptors using co-treatment with MT7 and AQ-RA 741. Our results show that only the M_2 subtype is present in the atria. In the ventricles, however, we were able to determine that 20% (on average) of the muscarinic receptors were subtypes other than M_2, with the majority of these belonging to the M_1 subtype. We were also able to detect a marginal fraction (6±2%) of receptors that, based on other findings, belong mainly to the M_5 muscarinic receptors. Co-treatment with MT7 and AQ-RA 741 was not a suitable tool for blocking of M_1–M_4 receptors and can not therefore be used as a method for M_5 muscarinic receptor detection in substitution to crude venom. These results provide further evidence of the expression of the M_1 muscarinic receptor subtype in the rat heart and also show that the heart contains at least one other, albeit minor, muscarinic receptor population, which most likely belongs to the M_5 muscarinic receptors but not to that of the M_3 receptors.

Keywords Heart · Immunoprecipitation · MT7 mamba toxin · Muscarinic receptors · Non-M2 muscarinic receptor · PLC activity

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Introduction

Muscarinic receptors belong to the G protein-coupled receptor family, and they transduce signals of the parasympathetic nervous system, i.e. they can reduce the heart rate. To date, five muscarinic receptor subtypes have been described and cloned. The odd-numbered subtypes (M_1, M_3, M_5) stimulate phospholipase C (PLC; via pertussis toxin-insensitive G_q protein), which cleaves phosphatidilinositolbisphosphate (PIP_2) to inositoltrisphosphate (IP_3) and diacylglycerol (DAG). The even-numbered subtypes (M_2, M_4) inhibit adenylyl cyclase (AC; via pertussis toxin-sensitive G_I protein), i.e. they decrease the amount of cyclic adenosine monophosphate (Moscona-Amir et al. 1989) and decrease the activity of protein kinase A (PKA).

It has long been believed that only one subtype is present in the mammalian heart—the M_2 receptor. This assumption was experimentally supported by a study with M_2 knockout

mice (Gomeza et al. 1999). However, the concept of the heart possessing a fully homogenous muscarinic receptor population has been challenged during the last decade (reviewed concisely by Dhein et al. 2001).

Studies based upon mRNA expression

There are many papers dealing with M_1–M_5 gene expression in the heart. Gallo et al. (1993) were first to report the presence of a non-M_2 mRNA (namely, M_1) in the mammalian heart, and the presence of mRNA for the other muscarinic acetylcholine receptor (mAChR) subtypes has been reported as well (Hassal et al. 1993; Hoower et al. 1994; Sharma et al. 1996; Colecraft et al. 1998; Shi et al. 1999; Hellgren et al. 2000; Oberhauser et al. 2001; Wang et al. 2001). Quantitative determination of mRNAs for all five mAChR subtypes has been recently reported by Krejčí and Tuček (2002).

Studies based upon radioligand binding/methods to measure receptor protein

One of the pioneering studies on muscarinic receptor subtype in the heart was reported in the strictly pharmacological paper of Yang et al. (1993). These researchers concluded that the second population of muscarinic receptors in the heart cells of rats is of the M_3 subtype. Shi et al. (1999) found (by electrophysiological and pharmacological methods) not only M_3, but also M_4 receptors in canine heart tissue, and Wang et al. (2001) demonstrated the presence of multiple subtypes (M_1, M_2, M_3 and M_5) in the human heart by immunochemistry and analysis of ligand binding. The minor population of heart muscarinic receptors and, in particular, the functional heart M_3 muscarinic receptors was discussed in review of Wang et al. 2004). In agreement with these results, Pérez et al. (2006) confirmed the presence of mRNA of the M_1–M_5 mAChR subtypes in both human cardiac atria and ventricles. Using immunoblotting methods, multiple antigen blot assay (MABA) and enyzme-linked immunosorbent assay (ELISA) with subtype-specific antibodies they also confirmed the presence of M_1, M_2, M_3, M_4 and M_5 proteins in membrane preparations from both atria and ventricles. These authors also suggest that muscarinic receptor subtypes undergo discrete transitions from a non-cooperative kinetics of non-interacting monomers to a cooperative kinetics of interacting oligomers. Luthin et al. (1988) found a small population of receptors in rat heart with a high affinity for pirenzepine. However, they showed that these [^{3}H]pirenzepine-labeled cardiac receptors were not precipitated by anti-M_1 antibody but by anti-M_2 antibody. They therefore concluded that rat heart contains pirenzepine-sensitive M_2 receptors. Luthin et al. (1988) also found that anti-M_1 antibody did not precipitate QNB-labeled receptor from rat heart.

Functional studies

As mentioned, the odd-numbered muscarinic receptors have been characterized primarily as stimulators of inositol metabolism. Consequently, evidence of increased phosphoinositide metabolism following cholinergic stimulation is one of the experimental findings supporting the conclusion that heart cells express more than one muscarinic receptor subtype (see Brown and Brown 1983; Moscona-Amir et al. 1989; Nadler et al. 1993; Sterin-Borda et al. 1995; Sun et al. 1996). Wang et al. (2004, 2007) reviewed the ever-increasing body of data indicating the presence of the M_3 muscarinic receptor and the functional consequences of the presence of this receptor in human heart. As these reviews were focused mainly on the human heart and the M_3 muscarinic receptor, the question of the existence of multiple subtypes existing in the heart tissue and of species differences arises. Ford et al. (1992) only found M_2 mRNA in the guinea pig heart, although they did observe stimulation of phosphoinositide metabolism that was not explainable by the properties of the M_2 receptors. In sheep, IP_3 synthesis was found to decrease with aging (Birk and Riemer 1992), and there was also a substantial decrease in M_2 mRNA. Pharmacological characterization revealed that the muscarinic population in sheep heart is consistent with the properties of M_2 and of M_3 or M_5 receptors. Data from functional studies of phosphoinositide metabolism changes in heart tissue and the data from the studies following ontogenetic aspects of muscarinic subtypes expression are still controversial: heart atria of rats express M_1 and M_2 receptors in newborn rats, while only M_2 are expressed in adult ones (Camusso et al. 1995; Borda et al. 1997). Nadler et al. (1993) described the stimulation of IP_3 synthesis in rat ventricles. Sterin-Borda et al. (1995) proposed that positive inotropy caused by carbachol in isolated atria is secondary, mediated via the PLC-NO pathway. Sun et al. (1996) concluded that the positive inotropic effect of carbachol in myocytes from rat heart ventricles is M_3 mediated. To support their conclusion, they reported the following experimental findings: (1) the increase in the force of contraction cannot be blocked by M_1 and M_2 antagonists but it can be blocked by the M_3 antagonist; (2) IP_3 accumulation is stimulated by carbachol and blocked by the M_3 antagonist; (3) stimulation of phosphoinositide hydrolysis is pertussis toxin-insensitive. In agreement with these findings, Trendelenburg et al. (2003, 2005) demonstrated, using M_2 and M_3 knockout mice, a heterogenous population of muscarinic receptors mediating the inhibition of sympathetic transmitter release (both M_2 and M_3 receptors affect the release). Similarly, Ponicke et al. (2003) demonstrated that the G_q coupled muscarinic receptor in the heart is of the M_3 subtype. They also found an increase in the heart rate after selective stimulation of these receptors. In contrast, Colecraft et al.

(1998) described the coupling of neonatal heart (ventricular) M_1 receptors to G_q, coupling to PLC.

In summary

To date, the nature of the minor heart muscarinic subtype remains unclear. The studies dealing with gene expression are limited by the fact that they are not able to detect the receptor protein expressed in the tissue, while the functional studies are typically limited by the fact that there is no specific ligand (agonist, antagonist) that is able to affect the specific receptor subtypes.

Moreover, there are a number of questionable points that need to be taken into consideration:

1) minor subtypes can differ in different species,
2) receptor expression and/or second messenger activation can change during ontogenesis,
3) the expression of the total number of muscarinic receptors differs in the heart atria and ventricles (a fact not taken into account in all studies).

Here we attempt to quantify muscarinic receptor subtypes—other than the M_2 subtype(s)— in the atria and ventricles of rat heart using different approaches:

1) set of competition binding experiments using three concentrations of antagonist per grade with pirenzepine, AFDX-116, 4-DAMP, PD 102807, *p*-F-HHSiD, DAU 5884, AQ-RA 741, methoctramine and tripinamide;
2) blockage of M_1 muscarinic receptors using MT7 toxin;
3) subtype-specific immunoprecipitation experiments; immunoprecipitation experiments are more sensitive than the Western blots because the former do not show protein expression as there is no binding to the appropriate ligand.
4) activation of PLC and using specific competitors (pirenzepine, DAU 5884 and AFDX 384) to block the effects of carbachol;
5) attempted blocking of M_1–M_4 receptors using incubation with AQ-RA 741 and MT7.

Material and methods

Reagents

[^{3}H]NMS ([methlyl-^{3}H]-*N*-scopolamine methyl chloride; specific activity 84 Ci/mmol) and [3H]QNB (L-quinuclidinyl [L-quinuclidinyl [phenyl-4-^{3}H]benzilate; specific activity 48 Ci/mmol) were obtained from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). AQ-RA 741 ((11-({4-[4-(diethylamino)butyl]-1-piperidinyl}acetyl)-5,11-dihydro-6H-pyrido(2,3-b)) was a generous gift from Boehringer Ingelheim (Biberach, Germany), p-F-HHSiD (para-fluoro analog hexahydro-sila-difenidol hydrocholride) was obtained from RBI (Natick, MA), pirenzepine (5,11-dihydro-11-([4-methyl-1-piperazinyl]acetyl)-6H-pyrido(2,3-b) benzodiazepin-6-one), methoctramine (methoctramine tetrahydrochloride *N*,*N*′-bis [6-[[(2-methoxyphenyl)-methyl] amino]hexyl]-1,8-octane diamiline tetrahydrochloride) and 4-DAMP (4-diphenylacetoxy-*N*-methylpiperidine methiodide) were from Sigma (St. Louis, MO), compounds DAU 5884 (8-methyl-8-azabicyclo-3-endo[3.2.1]oct-3-yl-1,4-dihydro -2-oxo-3(2H)-quinazolinecarboxylic acid ester hydrochloride) and AFDX 384 (*N*-[2-[2-[(Dipropylamino) methyl]-1-piperidinyl]ethyl]-5,6-dihydro-6-oxo-11H-pyrido [2,3b] [1,4]benzodiazepine-11 -carboxamide) were from Tocris (Bristol, UK), compound PD 102807 ([carboxyethyl, methyl, 9-O-methyl)]benzoxalazine isoquinoline) was a generous gift from Parke-Davis Pharmaceutical Research, tripinamide (tripinamide dihydrochloride) was a generous gift from Prof. C. Melchiorre, MT7 (muscarinic toxin 7, green mamba toxin) was from the Peptide Institute, (Osaka, Japan), AFDX-116 ([11-[12-diethylamino-methyl]-1-piperidinil]acetyl]-5-11-dihydro-6H-pyrido-2-3-b][1,4] benzo-diazepie-6-one]) was generous gift from Prof. H. Ladinsky. Antibodies to muscarinic receptors were purchased as follows: anti-M_2 (M9558) from Sigma-Aldrich (Czech Republic), anti-M_3 (sc-9108), anti-M_4 (sc-9109) and anti-M_5 (sc-9110) from Santa Cruz Biotechnology (Santa Cruz, CA), anti-M_1 from Alomone Labs (Jerusalem, Israel) (AMR-001, lot AN-01), Santa Cruz Biotechnology (sc-9106, lot A140) or Sigma-Aldrich (M9808, lot 90 K1372). All anti-receptor antibodies used were from rabbit. Goat anti-rabbit Ig (R5506) and donkey anti-goat Ig (G7767) were obtained from Sigma-Aldrich.

Animals and preparation of homogenates

Experiments were performed on juvenile male Wistar rats aged 46–55 days. The animals were handled in accordance with the legislature of the Czech Republic and EU Guidelines, and the experimental protocol was approved by the Committee for the Protection of Experimental Animals of the Institute of Physiology. The animals were sacrificed by cervical dislocation and decapitation. The cardiac atria and left and right ventricles were isolated, connective tissue was carefully discarded and the heart tissue was weighed, cut into small pieces and homogenized with an UltraTurrax homogenizer (Janke and Kunkel, Staufen, Germany) in ice-cold medium consisting of 20 mmol l^{-1} Na-HEPES (pH 7.4) and 1 mmol l^{-1} $MgCl_2$. The homogenates were filtered through medical gaze and centrifuged for 10 min at 600 *g* at 4°C. The sediments were resuspended in homogenization medium (the same as before) and washed twice by centrifugation (10 min at 600 *g*). The supernatants were

pooled and re-homogenized. The homogenates were stored frozen at −60°C until the measurements of radioligand binding.

Cell lines

Chinese hamster ovary (CHO) cells stably transfected with the human genes for muscarinic m_1–m_5 receptors were provided by Dr. M. Brann, University of Vermont Medical School, Burlington, VT. The cells were grown in plastic dishes in Dulbecco's modified Eagle's medium with 10% calf serum and 0.005% Geneticin. They were harvested by mild trypsinization 7 days after subculturing and washed twice by centrifugation (3 min at 300 *g*).

Competition binding studies on muscarinic receptors

Competition binding studies were performed using [^{3}H]NMS as a ligand in a modified procedure of Mysliveček et al. (2003). Homogenates corresponding to 2 or 5 mg fresh tissue per tube were incubated for 120 min at 25°C in a total volume of 1 ml. The incubation medium contained NaCl (136 mmol l^{-1}), KCl (5 mmol l^{-1}), $MgCl_2$ (1 mmol l^{-1}), Na-phosphate (pH 7.4, 1 mmol l^{-1}), Na-HEPES (pH 7.4; 10 mmol l^{-1}), phenylmethylsulfonyl fluoride (0.1 mmol l^{-1}) and [^{3}H]NMS (1800 pmol l^{-1}). The non-specific binding of the radioligand was measured in tubes to which atropine (5 μmol l^{-1} final concentration) had been added before the start of the incubation. The incubation was arrested by dilution with ice-cold distilled water, followed by rapid filtration through Whatman GF/B glass fibre filters in a Brandel cell harvester. The filters were washed with distilled water, and the retained radioactivity was measured by liquid scintillation spectrometry. The range of the concentrations of antagonists used is given in the Results. In general, we used three different concentrations of antagonist per grade.

Radioligand binding studies with MT7

M_1-receptor-selective toxin (MT7, m1-Toxin1) from green mamba (*Dendroaspis angusticeps*) venom has an extremely high selectivity for the M_1 subtype (Adem et al. 1988; Max et al. 1993; Olinas et al. 2000; Carsi and Potter 2000). Two different approaches were employed:

1) the binding of [^{3}H]NMS (2 nmol l^{-1}) to homogenates preincubated 30 min with MT7 (concentration range: 10^{-11}–10^{-7} mol l^{-1});
2) saturation binding experiments with increasing concentrations of [^{3}H]NMS (0.125–4 mol l^{-1}) in tissue preincubated 30 min with MT7 (10^{-8} mol l^{-1}).

Homogenates corresponding to 2 or 5 mg fresh tissue per tube were preincubated for 60 min at 38°C in a total volume of 1 ml. Incubation with [^{3}H]NMS followed for 60 min. The incubation medium contained NaCl (136 mmol l^{-1}), KCl (5 mmol l^{-1}), $MgCl_2$ (1 mmol l^{-1}), Na-phosphate (pH 7.4; 1 mmol l^{-1}), Na-HEPES (pH 7.4; 10 mmol l^{-1}), phenylmethylsulfonyl fluoride (0.1 mmol l^{-1}). The non-specific binding, termination of incubation and radioactivity measurements were made as stated before.

Determination of M_5 muscarinic receptors

The determination of the M_5 muscarinic receptor was performed using a modified method of Reever et al. (1997). These authors pre-treated tissue in 30 μg ml^{-1} crude green mamba toxin and 1 μmol l^{-1}AQ-RA741 to block all muscarinic receptors except for than M_5. This procedure blocked 99% of the M_1, M_2, and M_4 receptors and 85% of the M_3 receptors, while sparing the majority of M_5 receptors. The remaining receptors were then measured by binding with [^{3}H]NMS. We adopted a similar procedure using MT7 toxin instead of crude venom. First, we have evaluated the binding of 0.5 nmol l^{-1} [^{3}H]NMS in CHO cells individually expressing the human muscarinic receptor subtypes (M_1–M_5). The tissue was preincubated for 60 min with MT7 toxin (10^{-6} mol l^{-1}) and AQ-RA 741 (10^{-6} mol l^{-1}) and then incubated for 60 min with [^{3}H]NMS. For comparison, the binding of [^{3}H]NMS to specific subtypes pre-treated with AQ-RA 741 (10^{-6} mol l^{-1}) only was also performed.

Membrane preparation, receptor labeling, solubilization and immunoprecipitation

Membranes from stably transfected CHO cells individually expressing the human muscarinic receptor subtypes (M_1–M_5) and from rat atria and ventricles were prepared by homogenization (Ultra-Turrax homogenizer, 2×20 s, 20,500 rpm, on ice in 10 mmol l^{-1} Hepes pH 7.4+ 250 mmol l^{-1} sucrose+2 mmol l^{-1} EDTA+1 mmol l^{-1} PMSF) followed by slow centrifugation (Hettich centrifuge, 5 min/800 rpm/4°C). Pellets from the first centrifugation were rehomogenized and spun again under the same conditions. Combined supernatants were centrifuged at a higher speed (Sorvall centrifuge, SS34 rotor, 30 min/ 15,000 rpm/4°C). The resulting sediments were resuspended in glass-teflon homogenizer in 10 mmol l^{-1} Hepes pH 7.4+1 mmol l^{-1}EDTA+1 mmol l^{-1} EGTA+0.2 mmol l^{-1}PMSF (protein concentration: 1–2 mg ml^{-1} for cell lines and 3–4 mg ml^{-1} for the tissue). Muscarinic receptors present in the membranes were radiolabeled with [^{3}H]QNB (a subtype-nonselective antagonist with a high affinity and very slow dissociation; 2 nmol l^{-1} for 2 h at 30°C). The samples were then cooled on ice and centrifuged (Sorvall centrifuge, SS34 rotor, 15,000 rpm/30 min/4°C). Receptors

from the pellet were solubilized with digitonin and sodium cholate (extraction in 10 mmol l^{-1} Hepes pH 7.4/1 mmol l^{-1} EDTA+1 mmol l^{-1}EGTA+1% digitonin+0.2% sodium cholate; 1 h on ice, centrifugation 15,000 rpm/30 min/4°C). The mAChR content in the membranes was quantified by incubation with [^{3}H]QNB (0.2 ml; saturation binding: 0.1–2 nmol l^{-1} QNB for 2 h at 30°C) followed by filtration through Whatman GF/B glass fiber filters. The concentration of solubilized receptors was determined by gel filtration on a G-25 Sephadex column (1×18 cm, equilibrated with 10 mmol l^{-1} Hepes pH 7.4+1 mmol l^{-1} EDTA+1 mmol l^{-1} EGTA+0.2% Triton X-100; 0.3 ml solubilized receptors applied; 0.5-ml fractions of the eluate measured by liquid scintillation spectrometry). Incubation in the presence of 5 μmol l^{-1} atropin served as a control for non-specific binding. Soluble receptors were immunoprecipitated by subtype-selective antisera; all manipulations were carried out in the cold room (4°C) or on ice: 1 ml extract (containing 0.2–0.8 pmol l^{-1} mAChR) was incubated overnight with 5 μl (1 μg) of subtype-selective antisera (rabbit non-immune serum served as the negative control). After 12–16 h of incubation, 5 μl of goat anti-rabbit immunoglobulin (Ig)G was added to the extract-antisera solution; this was followed 4 h later with 10 μl of donkey anti-goat IgG. The solution was allowed to co-precipitate overnight. The samples were then centrifuged (Hettich centrifuge, 12,000 rpm/10 min/4°C), the immunoprecipitate were washed 3×1 ml 10 mmol l^{-1} Hepes pH 7.4+0.1% digitonin+0.02% sodium cholate, dissolved in 1% sodium dodecyl sulfate (SDS) (0.2+0.2+0.1 ml) and measured by liquid scintillation spectrometry.

Phospholipase C activity

Phospholipase C activity was measured using the enzymatic assay described by Dwivedy and Pandey (1999). Briefly, the tissue was homogenized on ice (10 mg of tissue per 100 μl of buffer) in 20 mmol l^{-1} Tris-HCl (pH 7.4), 2 mmol l^{-1} EGTA, 5 mmol l^{-1} EDTA, 1.5 mmol l^{-1} pepstatin, 2 mmol l^{-1} leupeptin, 0.5 mmol l^{-1} phenylmethylsulfonylfluoride, 0.2 U ml^{-1} aprotinin, and 2 mmol l^{-1} dithiothreitol using two short pulses of 10 s with a 30 s pause between pulses. For the assay, 5 μg of protein per tube was used. The tissue was incubated in incubation buffer (20 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} $CaCl_2$ and 100 mmol l^{-1} KCl, pH 7.4) containing 10 mmol l^{-1} LiCl, PIP_2 substrate (50 μmol l^{-1} unlabeled PIP_2), 2.0 mCi ml^{-1} [^{3}H]PIP_2 and 0.5 mg ml^{-1} cetrimide) in a total volume of 100 μl at 37°C (Thermoblock Biometra T1) for 40 min with buffer (basal activity) or with the addition of carbachol (1 mmol l^{-1}), carbachol + pirenzepine (1 μmol l^{-1}), carbachol + DAU 5884 (1 μmol l^{-1}) or carbachol + AFDX 384 (10 μmol l^{-1}). The reaction was terminated by the addition of 500 μl of 1 *M* HCl and 500 μl of a mixture of chloroform/methanol (1:1 vol/vol). The tubes were vigorously mixed and centrifuged at 1000 *g* for 10 min. The aqueous (upper) phase was transferred to a scintillation vial containing scintillation fluid, and the radioactivity counted. Each experiment had its blank in which the protein suspension was added after the reaction had been stopped with chloroform/methanol.

Data treatment

Radioligand binding data were treated as described previously (Mysliveček et al. 2003) with the use of GRAPHPAD PRISM ver. 5.01 (GraphPad Software, San Diego, CA) program. In detail, the curves were fitted using non-linear regression. The goodness of fit was determined using R^2, that was determined using the equation $R^2=1-SS_{reg}/SS_{tot}$, where SS_{reg} is sum of the squares of the distances of the points from the best-fit curve determined by nonlinear regression, and SS_{tot} is sum of the square of the distances of the points from a horizontal line through the mean of all *Y* values. The decision of preferred model was based on Akaike's information criterion (AIC; Akaike 1974) comparing one-site competition and two-site competition. Protein determination was performed using Peterson's modification of Lowry's method. Statistical significance of differences between means was evaluated with an unpaired two-tailed Student's *t*-test.

Results

Competition binding with muscarinic antagonists

Atria

Preliminary saturation binding experiments (n=3) with [^{3}H]NMS revealed the density of receptors to be 778.2±25.63 fmol mg^{-1} protein and the affinity (K_D) to be 1.10±0.10 nmol l^{-1}. The competition binding experiments with pirenzepine (10^{-10}–10^{-3} *M*) revealed one binding site in the heart atria [p*KI*=6.14±0.04, *nH* (Hill coefficient for binding of the radioligand) = 0.97±0.1]. Similarly, the competition with AFDX-116 (concentration range: 10^{-9}–10^{-4} *M*) showed a p*KI*=7.50±0.09 (*nH*=0.94±0.08). Finally, the competition with 4-DAMP (10^{-11}–10^{-5}) revealed only one binding site (p*KI*=7.81±0.03; n*H*=0.93±0.06). These results correspond to a uniform (M_2) muscarinic receptor population. The curves are shown in Fig. 1.

Ventricles

Preliminary saturation binding experiments (n=3) with [^{3}H]NMS revealed that the densities of receptors in the left and right ventricles were 290.2±4.4 and 367.9±8.9 fmol mg^{-1}

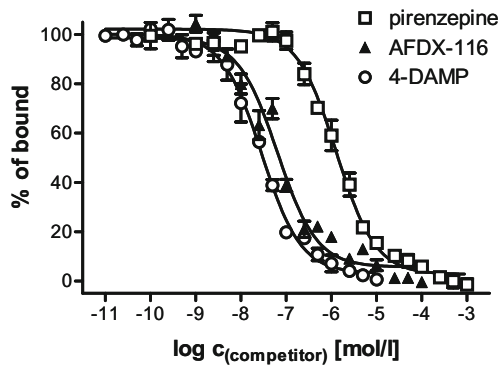

Fig. 1 The competition binding of [^{3}H]NMS and different antagonists in heart atria. Data are from three independent experiments in which tissue from three to four animals was pooled. Data are presented as means ± standard error of the mean (SEM). *Abscissa* Common logarithm of competitor concentration in mol/l, *ordinate* percentage of total bound

protein, respectively. The K_D was 1.43±0.06 and 1.01± 0.15 nmol l^{-1}, respectively. The competitions were carried on whole ventricles only, without differentiating the left and right ventricles. These competition-binding experiments with pirenzepine (10^{-10}–10^{-3} *M*) revealed two binding sites. Similarly, the competition with AFDX-116 (concentration range: 10^{-9}–10^{-4} *M*), 4-DAMP (10^{-11}–10^{-5} *M*), DAU 5884 (10^{-11}–10^{-6} *M*) and AQ-RA 741 (10^{-10}–10^{-5} *M*) showed two binding sites. In contrast, competitions with PD 102807 (10^{-9}–10^{-4} *M*), p-F-HHSiD (10^{-9}–10^{-4} *M*), tripinamide (10^{-10}–10^{-4} *M*) and methoctramine (10^{-10}–10^{-5} *M*) revealed one binding site. The data (pKI_1 or p*KI*, pKI_2 and fraction 1) are summarized in Table 1. The curves are shown in Fig. 2.

Subtype-specific immunoprecipitation experiments

Labeling of mAChR with [^{3}H]QNB was highly efficient (approaching complete saturation) and stable. The yield of mAChR solubilized from membranes was 40–55%, depending on the concentration of the protein. Dissociation of the label and/or degradation of receptors in solubilized receptor preparations were assessed by gel filtration. We found that 80–90% of the initial activity of the solubilized preparation (as measured immediately after solubilization) was still present in the extracts after 90 h under the conditions used for immunoprecipitation. The efficiencies of the receptor-specific antisera to immunoprecipitate the respective receptor protein were quantitated using receptors prepared from appropriate CHO cell lines. The results are shown at Fig. 3. At the antibody concentration used for the experiments (5 μl ml^{-1}), the efficiency of immunoprecipitation was 41% for anti-M_2 serum, 32% for anti-M_3, 45% for anti-M_4 and 50% for anti-M_5. We also tested anti-m_1 antisera from Alomone, Santa Cruz Biotechnology and Sigma (lot numbers given in Reagents), but all showed precipitation efficiencies that were too low to be applicable in our experiments. The specificities of antisera were confirmed by measuring the precipitation of comparable amounts of cloned mAChR subtypes. For heart tissue, the potential cross-reactivity of antibodies against M_2 receptors is of particular importance. The selectivities of individual antisera, as established by the immunoprecipitating receptor extract from m_2-transfected CHO cells, are illustrated in Fig. 4a: immunoprecipitation by anti-M_3, M_4 and M_5 was at the level of non-specific binding (control nonspecific trapping with non-immune serum). In a number of other cases, our antisera did not show cross-reactivity. It is also important to note that CHO cells themselves express different M receptor subtypes. Our assessment of the sensitivity of the immunoprecipitation is based on the following parameters and criteria. We were using 18,000–60,000 dpm specifically bound in tissue extracts. Non-specific precipitation represents 1.4–1.6% of total labeled receptors. Standard deviations (SD) of blanks were 30–70 dpm (for triplicates, depending on total radioactivity;

Table 1 Affinity (p*KI*) and the Hill coefficient for binding of the radioligand (*nH*) from binding experiments in the ventricles

Competitor[a]	Fraction 1	pKI_1 or p*KI*	pKI_2	*nH*
Pirenzepine	0.13±0.05	8.76±0.26	6.28±0.09	0.63±0.04
AFDX-116	0.86±0.004	7.78±0.02	5.65±0.11	0.78±0.04
4-DAMP	0.20±0.01	9.23±0.65	7.92±0.16	0.73±0.07
DAU 5884	0.35±0.02	9.81±0.11	7.23±0.07	0.47±0.02
AQ-RA 741	0.81±0.11	8.24±0.16	6.78±0.88	0.79±0.05
PD 102807	–	6.19±0.29	–	0.90±0.08
Tripinamide	–	7.73±0.09	–	1.15±0.12
Methoctramine	–	7.82±0.11	–	1.48±0.13
p-F-HHSiD	–	5.92±0.07	–	1.11±0.08

Data are means from three to five independent experiments in which tissue from three to four animals was pooled. The data are expressed as means ± standard error of the mean (SEM). These data are averaged values form the above-mentioned number of independent experiments. Note, that fraction 1 does not mean the major population in all cases

[a] See Reagents for the definitions of the competitors

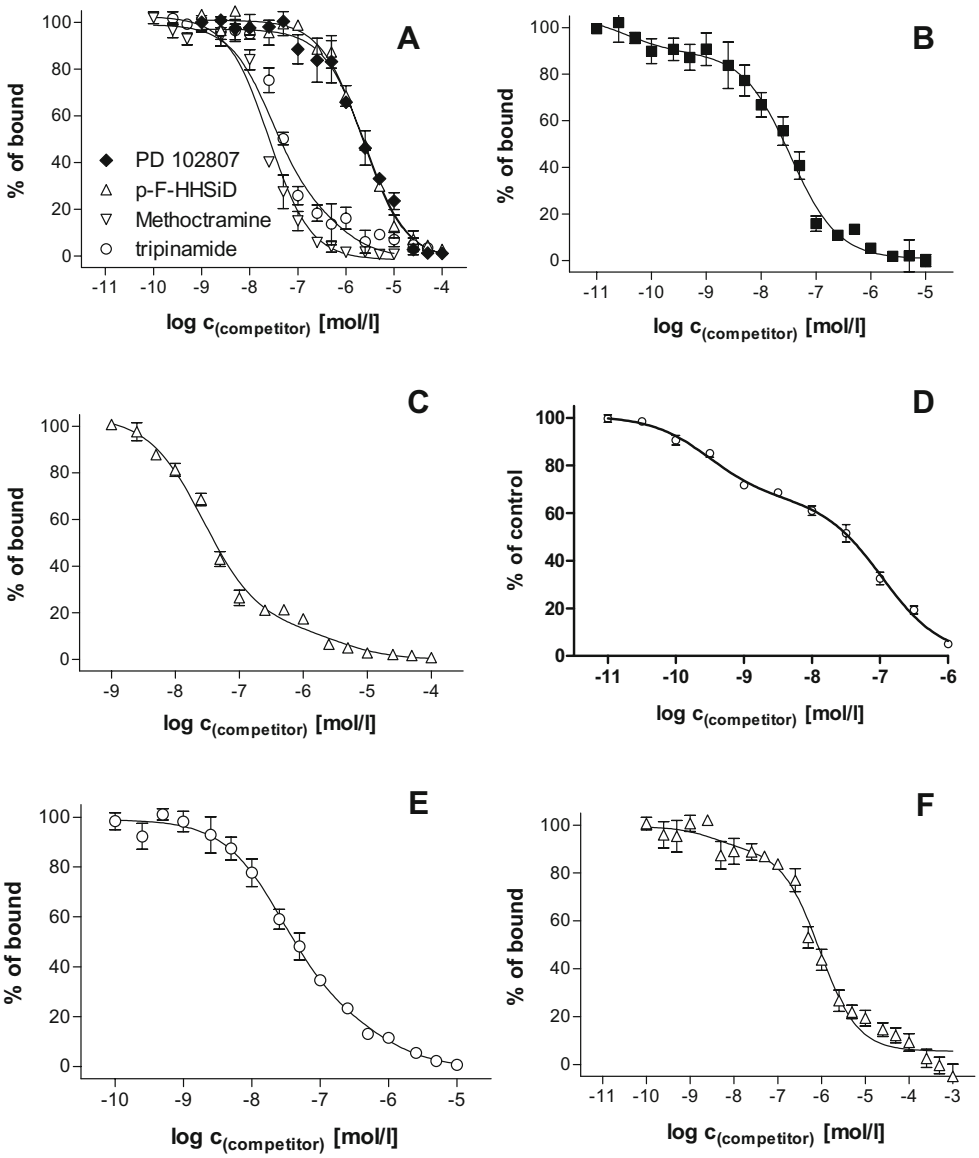

Fig. 2 The competition binding curves [^{3}H]NMS and different antagonists in heart ventricles. Data are from three to five independent experiments in which tissue from three to four animals was pooled. Data are presented as the means ± SEM. **a** The competition binding curves of antagonists which revealed one binding site only, **b–f** The competition binding curves of antagonists that revealed two binding sites: **b** competition with 4-DAMP, **c** competition with AFDX-116, **d** competition with DAU 5884, **e** competition with AQ-RA 741, **f** competition with pirenzepine. *Abscissa* Common logarithm of competitor concentration in mol/l, *ordinate* percentage of total bound

coefficient of variation 0.07–0.15). Specific signals representing threefold SD was considered to be the statistically correct limit of detection. We would be able to detect 90 dpm specifically precipitated from 18,000 dpm and 210 dpm precipitated from 60,000 dpm. Taking into account a precipitation efficiency of 32% (anti-M_3 antibody), we arrive at 0.4–1.6% of total receptors as the detection limit of our procedure (1.6% being the worst-case scenario). The preparations used in our experiments contained 0.35–0.5 pmol mAChR mg^{-1} protein for atria and 0.15–0.2 pmol mg^{-1} protein for ventricles. Illustrative results with immunoprecipitations from the left ventricular extract are shown at Fig. 4b. Combined estimations, expressed as the percentage of receptor subtypes precipitated by subtype-specific antibody (corrected for precipitation efficiency) from the three parts of the rat heart are listed in Table 2. Our anti-M_2 antibody precipitated 96±8%

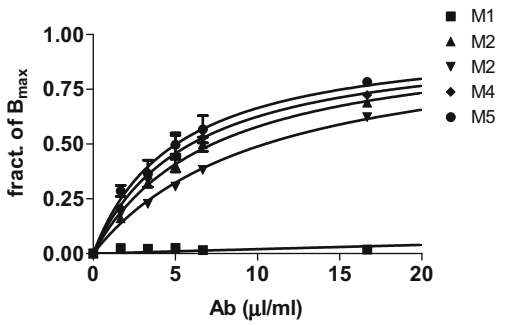

Fig. 3 Efficiency of immunoprecipitation (determined with receptors isolated from Chinese hamster ovary (CHO) cells transfected with the appropriate mAChR subtype). At concentration 5 µl ml^{-1} (concentration used for heart receptor immunoprecipitation), anti-M_2 antibody precipitated 41%, anti-M_3 32%, anti-M_4 45% and anti-M_5 50% of the receptors. This was only case in which [^{3}H]QNB was used as the radioligand

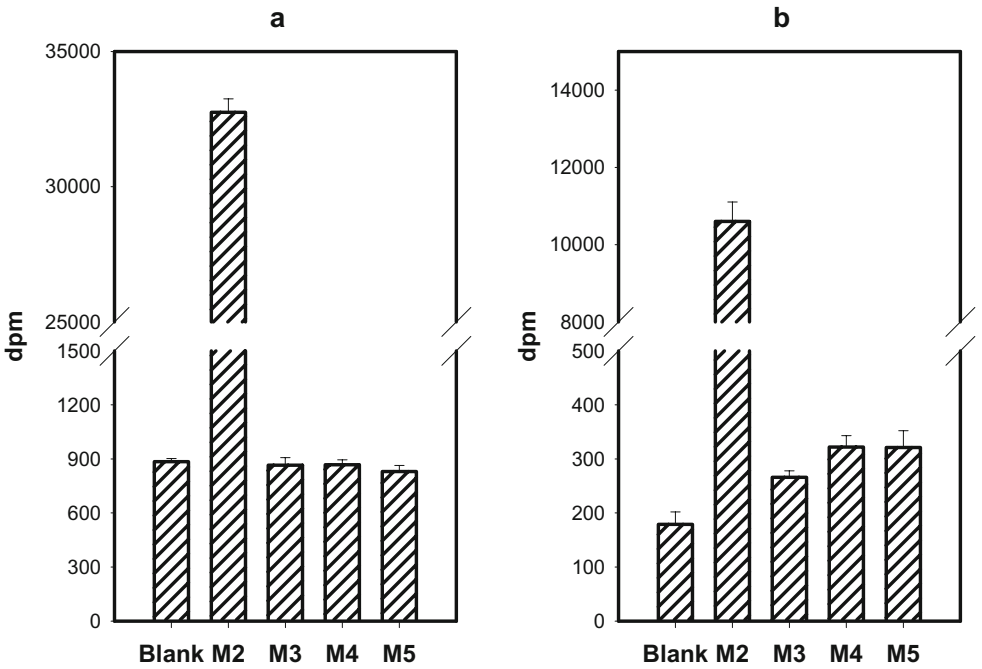

Fig. 4 **a** Selectivities of individual antisera were examined by using receptor extract from m_2-transfected CHO cells. Non-immune rabbit serum was used as to determine non-specific binding. The precipitation of M_2 receptors by anti-M_3, M_4 and M_5 was at the level of non-specific binding. **b** Representative experiment with immunoprecipitation of individual receptor subtype from the left ventricle. ^{3}H-QNB was used as radioligand (as this experiments served as control to immunoprecipitation experiments)

of labeled receptors, anti-M_3, M_4 and M_5 antisera showed low but detectable specific precipitation of their respective receptors which was close to the detection limit of our procedure. The anti-m_1 antisera available to us (from Alomone, Sigma and Santa Cruz Biotechnology, respectively) were not suitable for quantitative estimation of the M_1 subtype.

Binding to homogenates preincubated with MT7

Atria

The preincubation of homogenates with MT7 caused no substantial decrease in the binding of [^{3}H]NMS at all concentrations (data not shown). These results correspond to the presence of a uniform (M_2) muscarinic receptor population.

Ventricles

The preincubation of homogenates with MT7 (10^{-11}–10^{-7} *M*) caused a concentration-dependent decrease in which the data were best fitted with the model containing two binding sites: one comprising 9±1%, with apparent pK_I=9.7 and the other with apparent pKI_2=6.84, consistent with a major M_2 muscarinic receptor population (Fig. 5). These results can indicate that the minor population of muscarinic receptors in heart ventricles belongs mainly to the M_1 subtype.

Saturation binding experiments in tissue preincubated with MT7

The comparison of the saturation experiments in presence and absence of MT7 are shown in Table 3. These experiments confirmed that in the heart atria there is no M_1 subtype. On the other hand both left and right ventricles showed decrease in [^{3}H]NMS binding after MT7 pre-treatment.

Competition binding experiments in tissue preincubated with MT7

The competition binding experiments with pirenzepine showed that there are two binding sites. Pre-treatment of

Table 2 Data on the immunoprecipitation of the M_1–M_5 subtypes (expressed as percentage of receptor subtype precipitated by subtype-specific antibody and corrected for differences in the efficiencies of individual antisera)

	M_1	M_2	M_3	M_4	M_5
Atria (%)	ND	103±5	0.4±0.14	0.7±0.24	0.5±0.31
Left ventricle (V)	ND	93±8	1.5±0.21	2.5±0.37	2.5±0.53
Right ventricle (V)	ND	95±7	1.1±0.2	0.6±0.12	0.6±0.18

ND, Not determined

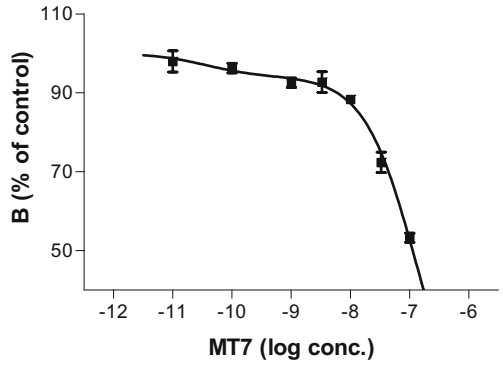

Fig. 5 Binding of [^{3}H]NMS to ventricular membranes preincubated with MT7 toxin. Data obtained from the fitted curve gave best fit with the two-binding site model: high-affinity (accounting for 9±1.1%) with pKI_{B1}=9.7 and low-affinity with pKI_2=6.84

the tissue with MT7 toxin abolished this biphasic binding in both the left (pKI=6.11±0.22; n=3) and right ventricles (pKI=6.37±0.06; n=3), clearly indicating a pKI for M_2 muscarinic receptors (see Table 4).

Determination of M_5 muscarinic receptors using the modified method of Reever et al. (1997) in CHO cells stably expressing M_1-M_5 muscarinic receptors

Co-incubation with both AQ-RA741 and MT7 toxin has been supposed to be a useful modification of Reever et al.'s method (1997) for M_5 muscarinic receptor determination. Therefore, we attempted to replace crude venom by MT7 toxin. In CHO cells stably transfected with human M_1–M_5 muscarinic receptor subtype, this procedure (MT7 toxin + AQ-RA 741) resulted in an 81% inhibition of binding in M_1 muscarinic receptors, 79% inhibition of binding in M_2 muscarinic receptors, 49% inhibition of binding in M_3 muscarinic receptors, 47% inhibition of binding in M_4 muscarinic receptors and 4% inhibition of binding in M_5 muscarinic (Fig. 6). Moreover, the comparison of binding in the presence of AQ-RA 741 only and the combination of drugs showed that the purified toxin behaves differently from crude the venom used by Reever et al. (1997): in the latter case the venom decreased the binding to all muscarinic receptor subtypes, while here MT7 decreased the binding to M_1 subtype only (see Fig. 6). Moreover, the addition of MT7 toxin also increased the binding to the M_3 and M_4 subtypes. Therefore, this method can not routinely replace the method of Reever for M_5 receptor determination.

Determination of M_5 muscarinic receptors in rat ventricles

Despite the fact that the combination of AQ-RA 741 and MT7 toxin can not be used for determining the M_5 muscarinic receptor subtype, we attempted to use this procedure to determine the "sub-minor" muscarinic receptor population that is probably masked in the amount of minor population based on the results of the competition binding experiments. Co-incubation of rat ventricles with both AQ-RA741 and MT7 toxin showed that there is 5.5±1.6% of muscarinic receptor subtypes that can not be blocked using this combination of drugs.

PLC activity

Carbachol was able to increase PLC activity (to 152% of control). This increase was not diminished by addition of the M_3 selective antagonist DAU 5884, but was inhibited by pirenzepine (M_1 antagonist) and AFDX 384 (M_5 antagonist). These data show that M_1 and M_5 receptors are able to affect PLC activity in the heart ventricles. All data are shown in Fig. 7.

Table 3 The densities and affinities of muscarinic receptors in heart atria and left and right ventricles pre-treated with MT7 or without pre-treatment (control)

Control		MT7	
Atria			
Bmax (fmol mg protein^{-1})	778.25±25.63	Bmax (fmol mg protein^{-1})	772.80±59.20
K_D (nmol l^{-1})	1.10±0.10	K_D (nmol l^{-1})	0,97±0,02
Left ventricles			
Bmax (fmol mg protein^{-1})	290.25±4.35	Bmax (fmol mg protein^{-1})	258.40±3.10 (89%)*
K_D (nmol l^{-1})	1.43±0.059	K_D (nmol l^{-1})	1.41±0.14
Right ventricles			
Bmax (fmol mg protein^{-1})	367.92±8.88	Bmax (fmol mg protein^{-1})	304.55±15.95 (83%)*
K_D (nmol l^{-1})	1.01±0.15	K_D (nmol l^{-1})	0.760±0.02

n=3–4

Percentage of control value is given in parenthesis; the asterisk (*) indicates when it is significantly different from control at $p<0.05$)

Table 4 Antagonist affinity constants (log affinity or p*KI* values) for muscarinic receptor subtypes

Antagonist	Receptor subtype				
	M_1	M_2	M_3	M_4	M_5
Pirenzepine	7.8–8.5	6.3–6.7	6.7–7.1	7.1–8.1	6.2–7.1
Methoctramine	7.1–7.8	7.8–8.3	6.3–6.9	7.4–8.1	6.2–7.2
4-DAMP	8.6–9.2	7.8–8.4	8.9–9.3	8.4–9.4	8.9–9.0
AF-DX 116	5.8–6.9	7.1–7.3	5.5–6.6	6.2–7.0	5.4–6.6
AF-DX 384	7.3–7.5	8.2–9.0	7.2–7.8	8.0–8.7	6.3
DAU 5884	9.40±0.04	7.40±0.05	8.80±0.03	8.50±0.02	Not known
Tripinamide	7.2–7.4	7.9–9.3	5.15–5.33	6.68–6.92	Not known
PD 102807	5.3–5.5	5.7–5.9	6.2–6.7	7.3–7.4	5.2–5.5
p-F-HHSiD	6.68–7.3	6.01–6.6	7.5–7.84	7.2	6.6–7.0
AQ-RA 741	7.6–7.8	8.21–8.9	7.4–7.5	7.9–8.2	5.8–6.1
MT7 toxin	9.8	<6	<6	<6	<6

Data were obtained from Caulfield and Birdsal 1998; Dhein et al. 2001; Doods et al. 1993, 1994; Eglen and Nahorski 2000; Choppin et al. 1999; Lazareno et al. 1998; Buckley et al. 1990; Bolognesi et al. 1998; Wang et al. 2004.

Discussion

Binding experiments

In general, the pharmacological characterization of the minor muscarinic receptor subtype presents some difficulties. There is no specific antagonist to one receptor subtype, except for mamba MT7 toxin (more than three orders of selectivity towards M_1 receptors). Therefore, we employed several approaches using MT7 toxin: (1) competition of heart tissue with MT7 toxin (decrease in binding means that there are M_1 receptors), (2) saturation binding after pre-incubation with MT7 toxin (if there is decrease in the Bmax, the minor M_1 population should be present) and (3) competition with pirenzepine after pre-incubation of tissue with MT7 toxin (if the M_1 receptor was present, then there should not be two population as all M_1 muscarinic receptors would be blocked, i.e. it would also prove that pirenzepine inhibits M_1 muscarinic receptors). It has also been shown previously that co-incubation of crude mamba toxin with AQ-RA 741 resulted in inhibition of all muscarinic subtypes except for M_5 (see Material and methods). Therefore, we tried to replace crude venom by MT7 toxin.

Our data shows that in the atria there is no other subtype than M_2. In the ventricles, we were able to identify certain amounts (13–35%, 19.6% on average) of non-M_2 muscarinic receptor subtype, which is the most probably the M_1 subtype. Also, a marginal fraction (about 5.5%) can be detected using a combination treatment consisting of MT7

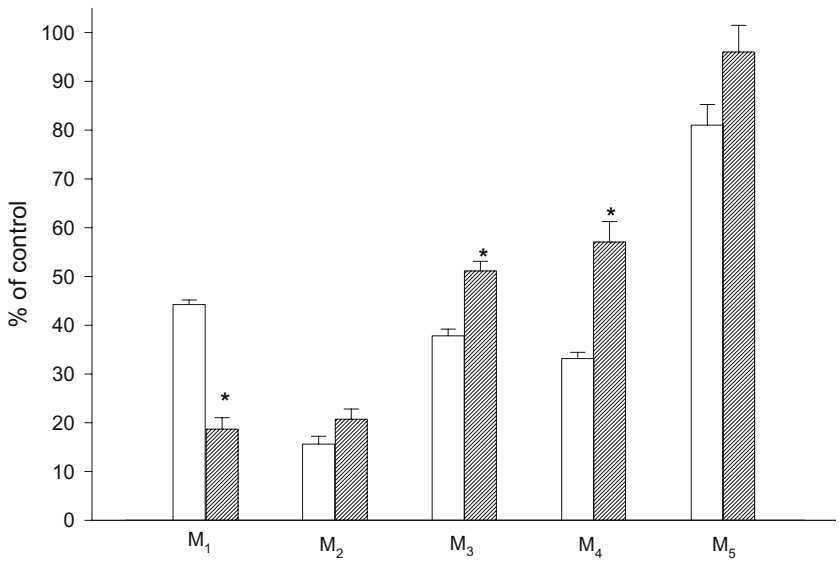

Fig. 6 Binding of [^{3}H]NMS to CHO cells transfected with appropriate human mAChR subtype. Comparison of preserved binding sites when incubated with AQ-RA 741 (10^{-6} mol l^{-1}) only [*left (empty) columns*] and with pre-treatment with AQ-RA 741 and MT7 toxin, as described in the Methods section [*right (shaded) columns*]. *$p<0.05$ difference between groups (AQ-RA 741 alone and combination of AQ-RA 741 and MT7 toxin)

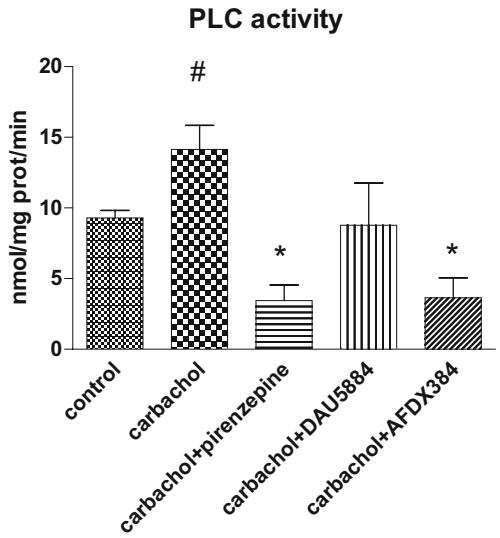

Fig. 7 Phospholipase C (*PLC*) activity in rat heart ventricles. *Ordinate* PLC activity expressed as nmol of IP_3 mg protein^{-1} min^{-1}. *$p<0.05$ indicates a difference from carbachol, #$p<0.05$ indicates a difference from control. The tissue was incubated for 40 min with buffer (basal activity) or with the addition of carbachol (1 mmol l^{-1}), carbachol + pirenzepine (1 μmol l^{-1}), carbachol + DAU 5884 (1 μmol. l^{-1}) or carbachol + AFDX 384(10 μmol l^{-1})

toxin and AQ-RA 741. It is possible to exclude the possibility that the minor subtypes are M_4 receptors (PD 102 807 did not show a biphasic curve). In addition, the possibility that the minor population belongs to M_3 receptors is rather improbable. There are more reasons why we draw this conclusion: (1) the competition with p-F-HHSiD showed a monophasic curve with p*KI*=5.92 that corresponds to the p*KI* in M_2 cloned receptors (6.01–6.6) but is inconsistent with the p*KI* for the M_3 subtype (7.5–7.84); (2) methoctramine and tripinamide, which have a low affinity for the M_3 muscarinic receptors (see Table 4) did not show biphasic curves; (3) DAU 5884, which is a highly functionally potent M_3 muscarinic antagonist, revealed pKI_1=9.81, which corresponds to the M_1 muscarinic receptors (p*K*=9.4, see Table 4) but not to M_3 (p*K*=8.8) and pKI_2=7.23, which correspond to the M_2 muscarinic receptors; (4) the competition and saturation binding experiments with almost the most specific drug for M_1 muscarinic receptors (MT7 toxin) clearly showed that the majority portion (9–17%) of the second muscarinic population belongs to the M_1 muscarinic receptors; (5) competitions with pirenzepine, AFDX-116 and 4-DAMP and competition with pirenzepine in tissue pre-treated with MT7 toxin showed that the minor population belongs to the M_1 subtype, but it is not possible to absolutely exclude any proportion of the M_5 subtype as the pKI_2 in competitions with AFDX-116 can correspond both to the M_1 and the M_5 subtype; (6) pKI_2 computed from AQ-RA 741 - [^{3}H]NMS competition lies between the estimated values of M_1, M_3 and M_5 receptors; therefore, we employed the method described in the Material and methods for identifying the M_5 receptors in order to be able to discriminate between the subtypes. Taken together, these data show that minor population (if it is up to 20%) represents binding to more receptor subtypes that can (as we show by functional experiments) contribute to cardiostimulating effects (increase in PLC activity) and, therefore, they protect the heart (like α_1-adrenoceptors; see Brodde et al. 2001).

Immunoprecipitations

Our data on immunoprecipitation confirmed that the population of M_2 receptors is not the only one in the heart ventricles, but their proportion in the total amount of receptors is very small (about 2.5% of M_5, 2.5% of M_4 and 1.5% of M_3 in the left ventricles and less than 1.1% of M_3 in the right ventricles). Also, these data confirmed our findings on the uniform muscarinic population in the atria. Unfortunately, the antibodies available to us were not able to detect the M_1 receptors. On the other hand, the immunoprecipitations also revealed that on the level of protein, there is another minor population of muscarinic receptor subtypes other than M_1. These data show that on the protein level the amount of M_5 and M_4 in the left ventricle is slightly higher than the amount of M_3 muscarinic receptor protein. Therefore, we cannot exclude the possibility that the heart minor muscarinic population is heterogeneous and consists of more than one muscarinic receptor subtype. This finding can be also supported by data from the competition binding studies with MT7 and AQ-RA 741.

Subtype-specific antibodies to muscarinic acetylcholine receptors (targeted to specific peptide sequences of all five subtypes) have been developed (Luthin et al. 1988; Levey et al. 1990, 1991; Wall et al. 1991a, b; Li et al. 1991; Mayanil et al. 1991; Yasuda et al. 1992) and used for quantifying muscarinic acetylcholine receptors by immunoprecipitation in a number of tissues in various species. In general, immunoprecipitation experiments are more sensitive than Western blots as they do not show protein expression despite the fact it does not bind to the appropriate ligand. Therefore, we employed this method instead of blotting. No cross-reactivity of the anti-M_3, anti-M_4 or anti-M_5 sera with the M_2 subtype was detected. Similarly to our data, Luthin et al. (1988) found that there was a small population of receptors in the rat heart with a high affinity for pirenzepine. However, these researchers showed that these [^{3}H]pirenzepine-labeled and also [^{3}H]QNB-labeled cardiac receptors were not precipitated by an anti-M_1 antibody but by an anti-M_2 antibody. They concluded that rat heart contains pirenzepine-sensitive M_2 receptors. These results

appear to negate our conclusion. On the other hand, it is improbable that other M_1-specific irreversible antagonist (MT7 toxin) can also detect pirenzepine-sensitive M_2 receptors. Therefore, these findings are more likely able to explain why these sites were not precipitated by an anti-M_1 antibody, i.e. it is more likely that these sites are immunoprecipitation pirenzepine-insensitive M_1 receptors. Hardouin et al. (2002), in contrast, did not detect QNB-labeled receptors in mouse heart and reported unchanged mAChR-mediated activation of PLC in the atria and ventricles of M_1-deficient mice, while Perez et al. (2006) detected all muscarinic receptor subtypes in the human heart using ELISA. However, the data from the latter study opens the question of whether this method should be used as it may lead to misinterpretation of the results given that in the atrium, septum and left and right ventricles these authors obtained a higher optical density for M_1 than for M_2 muscarinic receptors. Nevertheless, this study presumes a very important fact—that muscarinic receptors can co-operate with each other to form hetero- and homooligomers. Taken together with the conclusions of Luthin et al. (1988) and Colecraft et al. (1998), the data reviewed by Wang et al. (2004, 2007) and our data, the concept of specific binding properties of minor muscarinic receptor population is one of the most probable explanations of the different results obtained in the heart tissue.

Co-incubation of tissue with AQ-RA 741and MT7

Although, as is stated elsewhere, the co-incubation of AQ-RA 741 with MT7 toxin did not result in blockage of M_1–M_4 muscarinic receptors (as was in the case of crude venom toxin), these results indicate that the majority of this "sub-minor" population belongs to M_5 muscarinic receptors. This population, which is resist to co-incubation of tissue with MT7 and AQ-RA 741, can not belong to the M_3 and M_4 muscarinic receptors (as revealed by the competition experiments). The remaining subtypes (M_1, M_2 and M_5) should have the ratio (see Fig. 6) 19:21:96. Therefore, it is possible to suggest when 5.5% of the binding sites are resistant to combination with MT7 and AQ-RA 741, 0.9% belong to the M_1 muscarinic receptor subtype, 0.8% to the M_2 subtype and 3.9% to the M_5subtype.

PLC activity assay

The results of our experiments show that carbachol increased the production of IP_3, which could be inhibited most of all by pirenzepine, suggesting the primary importance of M_1 muscarinic receptors in phosphoinositide metabolism. On the contrary, DAU 5884, which is considered to be a functionally highly selective M_3 muscarinic antagonist, failed to inhibit the carbachol action. Inhibition of the carbachol effect was also recorded with 10^{-5} mol l^{-1} AFDX 384, i.e. the concentration that is able to block M_5 muscarinic receptors. These data are in part in agreement with the results of Dobrev et al. (2002) who were able to functionally characterize both M_1 and M_3 muscarinic receptors in human heart atria.

General discussion

Our data are in good agreement with that of Colecraft et al. (1998) who identified the M_1 receptors in functional and single-cell RT-PCR mRNA assays on neonatal rat ventricular myocytes. Yang et al. (1993), however, found the M_3 muscarinic receptors as the minor heart subtype, and Wang et al. (2004, 2007) have also reviewed this subtype as a probable minor subtype. The findings that favor the presence of M_3 muscarinic receptors are those of Ponicke et al. (2003). On the other hand, some findings have shown that human atria appear to possess both M_1, M_3 and M_5 receptors (Wang et al. 2001; Willmy-Matthes et al. 2003). There is no mRNA for M_3-receptors in rat atrial myocytes (single-cell PCR; Meyer et al. 2001). Moreover, Fisher et al. (2004) clearly demonstrated that heart rate responses remained unchanged in M_3 receptor-deficient mice, whereas bronchoconstrictor responses were totally abolished in these animals. These findings also question the role of the M_3 muscarinic receptor subtype in the regulation of the heart rate. On the other hand, Krejčí and Tuček (2002) found about 100-fold less mRNA for M_1 muscarinic receptors than for the other minor subtype. These results are in antinomy to that of Colecraft et al. (1998) who identified no other minor mRNA than M_1 in rat heart ventricular myocytes. The explanation of this disparity could lie in the fact that Colecraft et al. employed myocytes, but Krejčí and Tuček used a mixture of both myocyte and non-myocyte cells. We have also recently identified differences in receptor expression in cardiac tissue with neuronal ganglia and tissue that is virtually free of neuronal cells (Mysliveček et al. 2004, 2006).

It is also necessary to state that species differences and disease state may be the key in resolving the controversy surrounding the existence of non-M_2 receptors in the heart. For example, data available for canine atria clearly show the absence of M_1 and the presence of M_2 through M_4, but there is uncertainty regarding M_5 (Wang et al. 2001, 2004, 2007). Data on the human atrium clearly show that M_1 is present at the protein level and is functional in regulating potassium channels (Dobrev et al. 2002). Although it cannot be excluded that M_1 receptors are expressed in a diseased atrium only, it appears that their existence is a general phenomenon, although M_1 is not present in all species (i.e. dog; see data of Wang's group.).

In addition to data on the minor muscarinic population in the rat heart, we have also demonstrated that MT7 toxin can not be used instead of crude mamba venom (Reever et al. 1997) in simple routine M_5 muscarinic receptor detection. The increase in receptor binding (see Fig. 6) in comparison to [^{3}H]NMS binding when following a pre-treatment with AQ-RA 741 only suggests positive allosteric binding or positive co-operative binding with MT7 toxin.

Taken together, all these data provide further evidence that there are minor muscarinic receptor subtypes in the heart. Also, based on the results using different approaches, it is possible to suggest that the expression and function of minor muscarinic receptor subtypes is subject to very complicated interrelationships.

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