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REVIEW

Functional M_3 muscarinic acetylcholine receptors in mammalian hearts

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In contrast to most peripheral tissues where multiple subtypes of muscarinic acetylcholine receptor (mAChR) coexist, with each of them playing its part in the orchestra of parasympathetic innervation, the myocardium has been traditionally considered to possess a single mAChR subtype. Although there is much evidence to support the notion that one receptor subtype (M_2) orchestrates myocardial muscarinic transduction, there is emerging evidence that M_1 and M_3 receptors are also expressed and are of potential physiological, pathophysiological and pharmacological relevance. Clarifying this issue has a profound impact on our thinking about the cholinergic control of the heart function and disease and approaches to new drug development for the treatment of heart disease associated with parasympathetic dysfunction. This review article presents evidence for the presence of the M_3 receptor subtype in the heart, and analyzes the controversial data from published pharmacological, functional and molecular studies. The potential roles of the M_3 receptors, in parasympathetic control of heart function under normal physiological conditions and in heart failure, myocardial ischemia and arrhythmias, are discussed. On the basis of these considerations, we have made some proposals concerning the future of myocardial M_3 receptor research.
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Keywords: Muscarinic acetylcholine receptor (mAChR); M_2 receptor; M_3 receptor; heart; I_{KM3}; atrial fibrillation

Abbreviations: AA release, arachidonic acid release; AC inhibition, adenylyl cyclase inhibition; AF, atrial fibrillation; APD, action potential duration; CHF, congestive heart failure; 4-DAMP, 4-diphenylacetoxy-*N*-methylpiperidine methiodide; I3, the third transmembrane domain of mAChR; I_K, delayed rectifier K^+ current; I_{KACh}, acetylcholine-activated inward rectifier K^+ current; I_{KM3}, M_3 receptor-mediated delayed rectifier K^+ current; I_{Kir}, inward rectifier K^+ current; mAChR, muscarinic acetylcholine receptor; PTX, pertussis toxin; *p*-F-HHSiD, hexahydro-sila-difenidol hydrochloride, *p*-fluoro analog; PI hydrolysis, phosphoinositide hydrolysis; PLA2, phospholipase A2; PLC, phospholipase C; PLD, phospholipase D; RT–PCR, reverse transcription–polymerase chain reaction; TMA, tetramethylammonium

Introduction

One major breakthrough in the field of the cholinergic nervous system is the discovery of multiple subclasses of muscarinic receptors, owing to the development of pharmacological probes and molecular cloning techniques. Cholinergic receptors are traditionally divided into nicotinic and muscarinic subclasses; muscarinic receptors are selectively activated by muscarine and blocked by atropine. Five muscarinic acetylcholine receptor (mAChR) subtypes have been identified genetically by means of molecular cloning, originally designated m_1, m_2, m_3, m_4 and m_5. Four of them (M_1, M_2, M_3 and M_4) have also been pharmacologically and functionally characterized in primary tissues (Hulme *et al*., 1990; Van Zwieten & Doods, 1995; Eglen & Watson, 1996). These structurally distinct subtypes have characteristic distributions, pharmacological (binding) profiles and physiological functions. In many tissues/cells, multiple subtypes of mAChR coexist, with each of them playing a role in parasympathetic innervation.

Approaches for discriminating mAChR subtypes

Development of mAChR subtype-selective antagonists has made it possible to pharmacologically discriminate different mAChR subtypes. To date, a handful of compounds have been available, which possess reasonable selectivities towards different mAChR subtypes. These include pirenzepine and several muscarinic toxins isolated from the venom of green mamba (MTx) for M_1 (Jerusalinsky *et al*., 2000; Nasman *et al*., 2000; Bradley *et al*., 2003; Mourier *et al*., 2003); methoctramine, AF-DX 116, AF-DX 384 and tripitramine for M_2; 4-diphenylacetoxy-*N*-methylpiperidine methiodide (4-DAMP) and hexahydro-sila-difenidol hydrochloride, *p*-fluoro analog (*p*-F-HHSiD) for M_3; tropicamide, himbacine, PD102807(28) (Bohme *et al*., 2002), and muscarinic toxins MT1 and MT3 (Jerusalinsky *et al*., 2000) for M_4. Table 1 summarizes the pharmacological properties of mAChR subtypes. It is noted

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Table 1 Pharmacological characterization of mAChR subtypes

Antagonists	M_1/m_1	M_2/m_2	M_3/m_3	M_4/m_4	M_5/m_5
Atropine	9.0	8.7	9.2	8.9	8.9
Pirenzepine	8.0	6.3	6.8	7.1	6.9
Methoctramine	6.7	7.7	6.1	7.0	6.3
AF-DX 116	6.9	7.2	6.6	7.0	6.6
AF-DX 384	7.3	9.0	7.2	8.7	6.3
Tripitramine	8.5	9.4	7.1	8.0	7.3
4-DAMP	9.2	8.1	9.2	8.5	8.9
p-F-HHSi D	7.3	6.6	7.7	7.2	6.7
Darifenacin	7.8	7.0	8.8	7.7	8.0
Tropicamide	7.2	7.3	7.4	7.8	ND
Himbacine	6.7	8.0	6.9	7.8	6.1
PD102807(28)	4.9	6.5	6.8	9.0	4.9
MT1	22^{++}		72	29^{--}	
MT2	$>600^{+}$	NB	1200	1890^{-}	800–1000+
MT3	1100	NB	NB	1.4^{---}	NB
MT4	$^{-}$	NB	NB	$^{-}$	NB
MT7	0.2^{---}	NB	NB	NB	NB
m2-Toxin					

Antagonist affinities are expressed as $-\log K_i$ and derived from radioligand binding studies with the compounds; ND: data not available. For MTs, the affinities also derived from radioligand-binding studies are expressed as nM concentration; $^{+}$: stimulatory; $^{-}$: inhibitory, more symbols indicate stronger effects; NB: no binding up to 2–20 μM. The values are adapted from Eglen & Nahorski (2000), Eglen *et al.* (1994), Lazareno & Birdsall (1993), and Lazareno *et al.* (1990), Shi *et al.* (1999a), Bradley *et al.* (2003), Mourier *et al.* (2003), Nasman *et al.* (2000), Jerusalinsky *et al.* (2000), Kornisiuk *et al.* (1995), Carsi *et al.* (1999), Carsi & Potter (2000).

Table 2 Signal transduction mechanisms of mAChR subtypes

Antagonists	M_1/m_1	M_2/m_2	M_3/m_3	M_4/m_4	M_5/m_5
PI hydrolysis	+++	+	+++	+	+++
AC inhibition	+	+++	–	+++	–
cAMP stimulation	+++	+	+++	–	+++
PLC stimulation with PI hydrolysis	+++	+	+++	+	+++
PLA2 stimulation with AA release	+++	$\pm$	+++	$\pm$	+++
PLD stimulation	+++	+	+++	+	+++
Increase in $[Ca^{2+}]_i$	+++	–	+++	–	Unknown

+++: strong effects; +: weak effects; –: no effects; $\pm$: weak effects when overexpressed, but no effects at physiologic levels. The results are adapted from Hulme *et al.* (1990) and Felder (1995).

that (1) there are no selective antagonists to any one of the subtypes; instead, the pharmacological profiles show varying extents of overlap among different subtypes and (2) there are at present no antagonists available with preferential affinity towards the M_5 receptor. Nonetheless, the selectivity of these compounds is sufficient to allow for discriminating different subtypes if their expression abundances are sufficiently high and sufficiently different in a given tissue/cell. Normally, to conclude the presence of a given mAChR subtype in a given tissue/cell, it is necessary to obtain evidence from several antagonists for the subtype of interest.

Great efforts have been made to understand the diverse array of responses mediated by mAChR activation, in terms of receptor and effector heterogeneity. Elucidation of the characteristic signaling mechanisms of various mAChRs allows us to functionally distinguish different subtypes. Activation of mAChRs is implicated in activation of several cellular signaling pathways, including adenylate cyclase, phospholipase C (PLC), phospholipase A2 (PLA2), phospholipase D (PLD) and intracellular Ca^{2+} signaling (Hulme *et al.*, 1990; Felder, 1995; Brodde & Michel, 1999). Table 2 highlights the signal transduction mechanisms associated with various subtypes of mAChRs. An inspection of Table 2 reveals both broad generalization and several notable exceptions. In general, the odd-numbered receptors M_1, M_3 and M_5 isoforms are characterized biochemically by stimulation of a large mobilization of inositol phospholipids mediated *via* a PTX-insensitive $G_{q/11}$-protein, while having a small stimulatory effect on adenylate cyclase activity. The even-numbered receptors M_2 and M_4 isoforms are linked to an inhibition of adenylyl cyclase activity *via* a PTX-sensitive G_i-protein and only a modest stimulation of phosphoinositide hydrolysis when overexpressed. The M_1, M_3 and M_5 receptors couple to PLC, PLA2 and PLD with higher efficacy than do the M_2 and M_4 receptors. In addition, the M_1, M_3 and M_5 receptors can stimulate a rise in intracellular Ca^{2+}. These differences help us roughly differentiate the functional subtypes of mAChR. The following caveats should be noted. First, a single mAChR might couple to more than one G protein (Haga *et al.*, 1990). There is now persuasive evidence that recombinant M_1, M_3 and M_5 receptors in cell lines can interact with G_s and G_i proteins (Eglen & Nahorski, 2000). Second, different subtypes of mAChR might couple to the same G protein (Hulme *et al.*, 1990; Felder, 1995; Brodde & Michel, 1999). Finally, a

Table 3 Physiological function of mAChR subtypes

Antagonists	M_1/m_1	M_2/m_2	M_3/m_3	M_4/m_4	M_5/m_5
Activation of I_{Kir}	−	+ + + (I_{KACh})	−	+ + + (GIRK1)	Unknown
Activation of I_K	−	−	+ + + (I_{KM3})	+ + + (I_{KACh})	Unknown
Inhibition of I_f	Unknown	+ +	Unknown	Unknown	Unknown
Inhibition of M current	+ + +	−	+ + +	−	Unknown
Inhibition of mitogenesis	+ +	+ +	+ +	Unknown	+ +
Stimulation of mitogenesis	+ +	+ +	+ +	Unknown	Unknown
Slowing of heart rate	−	+ + +	+	±	Unknown
Shortening of cardiac action potentials	−	+ + +	+ +	Unknown	Unknown
Cardiac contraction	↑↑	↓↓	↑	Unknown	Unknown
Smooth muscle contraction	Unknown	↑↑	↑↑↑	Unknown	Unknown

+ + +: strong effects; +: weak effects; −: no effects; ±: facilitating effects; ↑: increase; and ↓: decrease. I_{Kir}: inward rectifier K^+ current; I_K: delayed rectifier K^+ current; I_f: pacemaker current.

receptor may couple to a singular signal transducer, but the effector response subsequently activated may vary according to the particular cell type.

Several physiologic outcomes produced by mAChR stimulation have also been frequently employed to help discriminating the subtypes functionally expressed in a given tissue/cell. These physiologic functions are summarized in Table 3. For instance, bradycardia has been considered as a hallmark of the M_2 function (Stengel *et al*., 2000), and stimulation of contraction force of several smooth muscles is the function predominated by the M_3 receptors (Pönicke *et al*., 2003). It should be noted that the function of the M_5 receptors has only been demonstrated in cell lines expressing recombinant receptors and in M_5-transgenic mice or M_5-deficient mice (Basile *et al*., 2002; Fink-Jensen *et al*., 2003). It is also important to bear it in mind that many of the functions assigned to a given subtype of mAChR, as listed, are based on commonly accepted idea yet to be confirmed and on meagre studies found in the literature, and should not be taken as a dogma for classifying different subtypes of mAChR.

Molecular biology approaches further enable us to distinguish the presence of transcripts and differential expression of various subtypes of mAChR in tissues/cells (Wess, 1996; Brodde *et al*., 2001; Hulme *et al*., 2003; Myslivecek and Trojan, 2003; van Koppen and Kaiser, 2003). With its high sensitivity, reverse transcription–polymerase chain reaction (RT–PCR) can be used to detect low-abundance transcripts. Antibodies for all the five different subtypes of mAChR have been commercially available and have been fairly successfully used for studying mAChR expressions at the protein level, though their specificities still await being rigorously verified. More detailed information on this subject can be found in several excellent review articles (Wess, 1996; 2003; Hamilton *et al*., 1998; Kostenis *et al*., 1998; Hulme *et al*., 2003).

In contrast to most peripheral tissues, the myocardium has been considered to possess a single mAChR subtype. The M_2 receptor has long been believed to be the only mAChR subtype in the heart (Bonner *et al*., 1987; Peralta *et al*., 1987; Brann *et al*., 1993; Van Zwieten & Doods, 1995). However, this 'homogeneous M_2 receptor expression' concept has been challenged; many recent studies have demonstrated the possible presence of non-M_2 receptors, particularly, the M_1 and M_3 receptors, in the heart. The 'heterogeneous muscarinic receptor expression' concept has emerged. On the other hand, there are also some recent studies arguing against the 'heterogeneous muscarinic receptor expression' concept and guard the 'homogeneous M_2 receptor expression' concept. The possibility that the M_3 receptors may play a role in the cholinergic control of the heart attracts increasing attention from the researchers. This review focuses on the issue regarding only the M_2 and M_3 receptors in the heart.

Evidence for heterogeneous myocardial muscarinic receptors

Despite the classical notion that the cardiac mAChR is of exclusively the M_2 subtype, there is evidence for a possible role of other subtypes, particularly the M_1 and M_3 receptors. Functional M_1 receptors have been identified and characterized in rat ventricular myocytes by Sheu's group (Sharma *et al*., 1996; 1997; Colecraft *et al*., 1998; Brodde and Michel, 1999) and in mouse right atria by Islam *et al*. (1998), in late 1990s. Early indications for the existence of a M_3 receptor stems from studies in rabbit (Jaiswal *et al*., 1989) and dog atria (Akahane *et al*., 1990), showing some physiological functions of mAChR with pharmacological profiles that do not fit to the known properties of the M_2 receptors but are better explained by the presence of the M_3 receptors. Similar disparity between the characteristic M_2 function and antagonist effects was also revealed in guinea-pig cardiac muscles and neonatal rat ventricular cardiomyocytes by several studies published in the early '90s. Recently in late '90s, pharmacological data from radioligand-binding studies have provided more favorable evidence in support of the view of the functional M_3 subtypes in hearts. Moreover, combined functional and molecular studies further support the presence of the cardiac M_3 receptors, with data indicating expression of the M_3 transcript and protein in the hearts of various species including mouse, rat, canine and man.

Pharmacological evidence

Different mAChR subtypes have their characteristic affinities for the binding of different mAChR antagonists (see Table 1). Yang *et al*. (1992) performed binding assays in membrane homogenates from dog left ventricular tissues. Their data demonstrated that pirenzepine competed with [^{3}H]QNB or [^{3}H]NMS for a single binding site with a K_i value of $\sim$0.2 and 0.6 μM, respectively, which is in agreement with the affinities to

the M_2 or M_3 receptors, but not the M_1 receptors. On the other hand, competition of [^{3}H]ligand binding with M_3-selective antagonists 4-DAMP and HHSiD, respectively, gave a best fit for a two-binding site model, favoring the existence of the M_3 subtype. The authors excluded the presence of M_1 receptors in the canine left ventricle and suggested the presence of a second population of mAChR distinct from the classical cardiac M_2 receptors.

Our laboratory conducted similar studies in membrane homogenates from canine atria (Shi *et al*., 1999a, b; Wang *et al*., 1999a). Displacement binding of [^{3}H]NMS in the presence of pirenzepine, methoctramine and 4-DAMP was analyzed with a two-site binding model (see Figure 1). The displacement of [^{3}H]NMS binding by pirenzepine does not discriminate the M_2, M_3 and M_4 subtypes, and is inconsistent with its affinity to an M_1 receptor. The high-affinity binding of methoctramine ($pK_i = 7.7$) suggests the existence of the M_2 and M_4 subtypes (Van Zwieten & Doods, 1995) and its low-affinity binding ($pK_i = 6.6$) identifies, but does not distinguish between, the M_3 and M_5 subpopulations. Similarly, 4-DAMP binding revealed two groups of mAChRs with high-affinity binding ($pK_i = 9.1$), in agreement with its affinity to the M_3 and M_1 receptors and low-affinity binding ($pK_i = 7.0$) for an M_2 subtype. However, with respect to pirenzepine binding, the high-affinity pK_i value for 4-DAMP binding would more likely correspond to an M_3 receptor. Taken together, the results from our binding experiments suggest the presence of M_3, in addition to M_2, subtypes of mAChRs in the canine atrium.

Similar results were seen with the membrane homogenates extracted from human atria and ventricles. Competition binding of [^{3}H]NMS with methoctramine and 4-DAMP yielded data consistent with the presence of the M_2 and M3 mAChRs in both human atrial and ventricular tissues. 4-DAMP binding also revealed two groups of mAChRs, with a high-affinity binding consistent with its affinity to the M_3 and M_1 receptors (Van Zwieten & Doods, 1995) and a low-affinity binding typical of 4-DAMP binding to M_2 receptors (Figure 1) (Wang *et al*., 2001).

In addition, we have also performed binding assays with three partial mAChR agonists tetramethylammonium (TMA), pilocarpine and choline (Hoss *et al*., 1990; Gabelt and Kaufman, 1992; Wess *et al*., 1992; Zakharov *et al*., 1993; 1995; Poyer *et al*., 1994; Kennedy *et al*., 1995) in both canine and human membrane preparations (Figure 1) (Shi *et al*., 1999a, b; Wang *et al*., 1999a; 2001). All the three agonists were able to displace in a competitive manner the binding of [^{3}H]NMS to mAChRs. The low-affinity K_d value of TMA binding (2.5 mM) is almost identical with the value for cloned M_3 receptor (2.2 mM) reported by Wess *et al*. (1992). However, it should be noted that the binding affinities of these partial agonists could also be interpreted as binding to the M_2 receptors.

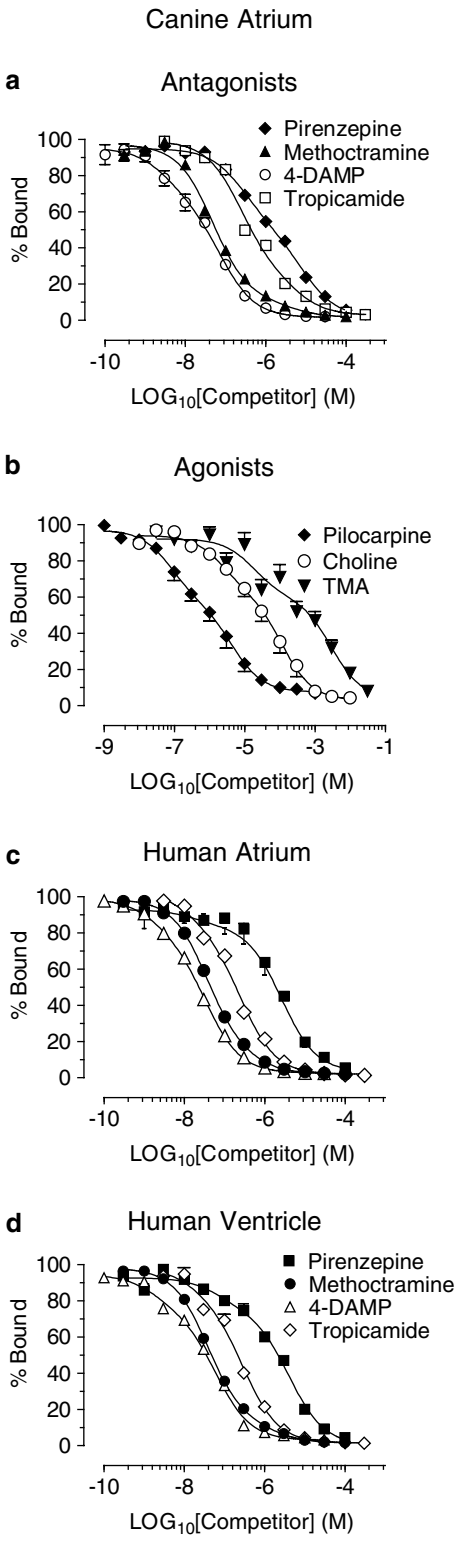

Figure 1 Displacement binding of [^{3}H]-NMS to membrane homogenates from canine atrial tissues (a, b), human atrium (c) and human ventricle (d) with various mAChR subtype-selective antagonists (pirenzepine for M_1, methoctramine for M_2, 4-DAMP for M_3 and tropicaminde for M_4) or with partial mAChR agonists (pilocarpine, choline and TMA). The competition-binding curves are all best fitted by the two-site binding model-yielding a high- and a low-affinity binding pK_i values (see text for description).

Functional evidence

Chassaing *et al*. (1984) reported a study on the chronotropic and inotropic effects of four agonists and three antagonists of mAChRs in isolated guinea-pig atria. Based on the observed differences in the potencies and efficacies of these compounds in terms of their effects on heart rate and contraction, they proposed that there are two functional cardiac mAChR subtypes: one mediating the regulation of heart rate and the

other contractile force. However, Clague *et al*. (1985) reassessed the actions of the agonists and antagonists on atrial rate and contraction, as compared with those in ileal contractions, of guinea-pigs. They found that the differences in agonist potencies in these two tissues were attributable to either differences in intrinsic efficacy or susceptibility to the action of acetylcholinesterase. The small differences in agonist potency observed between atrial and ileal muscarinic receptors were considered not sufficient to indicate receptor heterogeneity.

The study reported by Jaiswal *et al*. (1989) provides the first evidence for the functional M_3 receptors in mammalian hearts. The authors demonstrated that, in the isolated rabbit heart, ACh increased prostaglandin synthesis and the effect was inhibited by a low concentration of 4-DAMP (10 nM). Although the investigators considered 4-DAMP as an M_2 antagonist, the concentration used would most likely block the M_3 receptors with minimal effects on the M_2 receptors. The same group (Kan *et al*., 1996) has later re-evaluated prostacyclin synthesis in rabbit hearts and now believes that ACh can function *via* the M_3 receptors in ventricular myocytes. They found that ACh-induced 6-keto-postaglandin (1 alpha) production in ventricular myocytes was reduced by HHSiD and AF-DX 116, but not by pirenzepine. Moreover, the decrease by ACh of isoproterenol-stimulated cAMP accumulation was minimized only by AF-DX 116, but not by HHSiD or pirenzepine. While pertussis toxin (PTX) abrogated the ACh-induced decrease in cAMP (consistent with the M_2 receptor-G_i protein coupling), it did not affect the ACh-induced prostaglandin synthesis (consistent with G_q protein coupling). These results are a strong indication of co-existence of the functional M_2 and M_3 receptors in rabbit ventricles.

It has been well established by several groups that mAChR agonists can evoke increases in IP formation in rat and guinea-pig cardiomyocytes (Ford *et al*., 1992; Sun *et al*., 1996; Pönicke *et al*., 2003); this is a typical response to stimulation of the M_1, M_3, or M_5 receptors, but not of the M_2 or M_4 receptors. Ford *et al*. (1992) analyzed mAChR-mediated PI hydrolysis in guinea-pig atria and ventricles. The actions of several antagonists including HHSiD and *p*-F-HHSiD generated an affinity profile skewed from the pure M_2 responses, suggesting 'a second population of muscarinic sites' (Ford *et al*., 1992). Sun *et al*. (1996) studied the antagonism of carbachol-induced chronotropy and IP accumulation in neonatal rat ventricular myocytes. They found that HHSiD blocked carbachol effects, while pirenzepine and AF-DX 116 had no effects. They concluded that neonatal ventricular myocytes have a heterogeneous population of muscarinic receptors including the M_2 and M_3 subtypes. More recently, Pönicke *et al*. (2003) provided further evidence for the presence of the functional M_3 receptors in adult rat ventricular myocytes. The authors assessed carbachol-induced IP formation in isolated myocytes. They found that the carbachol-induced IP formation was significantly enhanced by pre-treatment with PTX and this effect was inhibited by darifenacin, an M_3-selective inhibitor (Smith & Wallis, 1997), with a pK_i value of 8.7, but was not affected by pirenzepine or AF-DX 116 and himbacine. The author concluded that there exists in adult rat cardiomyocytes the M_3 subtype that is coupled to activation of the PLC/IP_3 pathway.

There is also functional evidence for the existence of the M_3 receptors in the mouse atrium. Nishimaru *et al*. (2000) found a biphasic response to ACh with a transient negative inotropic response followed by a positive inotropic effect in isolated mouse atria, which could both be inhibited by atropine. Detailed analysis revealed that the negative inotropic response could be antagonized by the M_2-selective antagonist gallamine and was sensitive to PTX, whereas the positive inotropic response was inhibited by the M_3-selective antagonist *p*-F-HHSiD and was insensitive to PTX, which is in support of the view that there is an M_3 subtype in mouse atria mediating positive inotropic effects induced by mAChR agonists (Nishimaru *et al*., 2000).

In cats, indication for coexistence of the functional M_1, M_2 and M_3 receptors has recently been documented (Osadchii, 2003). The author investigated the influence of mAChR subtype-selective inhibitors on the ECG P–P interval in response to vagal bursts and he found that block of the M_1 (pirenzepine), M_2 (metoctramine and gallamine) or M_3 (4-DAMP) cholinoreceptors diminished vagally induced minimal and maximal prolongation of the ECG P–P interval. In another study in anesthetized cats, the author (Koss, 1997) found that 4-DAMP antagonized the bradycardia induced by vagal stimulation.

Yang *et al*. (1992) compared the inhibition of carbachol-mediated PI hydrolysis by pirenzepine, AF-DX 116 and 4-DAMP in dog left ventricular cells and obtained an affinity profile dissimilar to the classical cardiac M_2 response. With isolated blood-perfused dog atria, Akahane *et al*. (1990) compared the inhibitory potency of carbachol-induced negative chronotropic and inotropic responses and found that the potency of 4-DAMP = atropine but > AF-DX 116 and >> pirenzepine, suggesting a role of the M_3 subtype. Recently, existence of the functional M_3 receptors in canine and guinea-pig hearts has also been documented by our laboratories (Shi *et al*., 1999a, b; Wang *et al*., 1999a). Our studies demonstrated that several nonselective mAChR agonists including choline (0.1–10 mM), pilocarpine (0.1–10 μM) and TMA (1 μM–10 mM) each can induce a same novel delayed rectifier K^+ current (we named it I_{KM3}, meaning the M_3 receptor-activated delayed rectifier K^+ current) in dispersed cardiomyocytes from guinea-pig and canine atria. Distinct from I_{KACh} that possesses inwardly rectifying property, I_{KM3} has a linear current–voltage relationship. I_{KM3} can be suppressed by 4-DAMP methiodide (2–10 nM), 4-DAMP mustard (4–20 nM) or *p*-F-HHSiD (20–200 nM), but not by antagonists to the M_1 (pirenzepine), M_2 (methoctramine) or M_4 (tropicamide) receptors, whereas I_{KACh} was inhibited by methoctramine, but not by the M_3-selective inhibitors. In fact, early in 1994, Fermini & Nattel (1994) first described the K^+ current activated by choline *via* the stimulation of mAChRs in canine atrial myocytes. Their data argued against the role of M_1 receptor subtype or nicotinic receptors in this function. Subsequently, Navarro-Polanco & Sànchez-Chapulam (1997) demonstrated that 4-aminopyridine (4-AP), a K^+ channel blocker, also activated a similar K^+ current in cat atrial cells, an effect requiring stimulation of mAChRs. As these currents possess biophysical properties distinct from I_{KACh}, novel subtypes of mAChRs other than the M_2 were proposed by these authors as a mechanism underlying the activation of these channels. Unfortunately, no further characterization in terms of mAChR subtypes was performed in these earlier studies.

Furthermore, our laboratories discovered that choline (0.1–10 mM) and pilocarpine (0.1–10 μM) both caused

significant slowing of heart rate and shortening of APD in guinea-pig atria (Wang *et al*., 1999a; Shi *et al*., 1999b). The effects were blocked only by 4-DAMP (2–10 nM) or *p*-F-HHSiD (20–200 nM) and could be reproduced in the presence of methoctramine to inhibit the M_2 receptors. Figure 2 illustrates the relationships between I_{KM3} induction, heart rate slowing and APD shortening, by pilocarpine and reversal by co-application with 4-DAMP in guinea-pig atrium. Intriguingly, TMA (0.5–50 mM) has also been reported to slow the sinus rate and to weaken the contraction of rat hearts (Zakharov *et al*., 1993; Kennedy *et al*., 1995). Our data on M_3-mediated I_{KM3} activation reveal a possible mechanism underlying, at least in part, the negative inotropic and chronotropic effects produced by choline, pilocarpine or TMA. These compounds activate I_{KM3} and cause membrane hyperpolarization and APD shortening, presumably due to the slow deactivation kinetics of I_{KM3} (Shi *et al*., 2003). Membrane hyperpolarization can result in weakening of automaticity, and thereby slowing of heart rate. On the other hand, APD shortening can indirectly decrease Ca^{2+} entry into the cell, which can in turn result in reduction of contractile force. It is noteworthy that the low-affinity bindings of choline, pilocarpine or TMA to mAChRs are quite compatible with the concentrations at which these compounds activate the K^+ current and alter heart rate and APD in both canine and guinea-pig hearts.

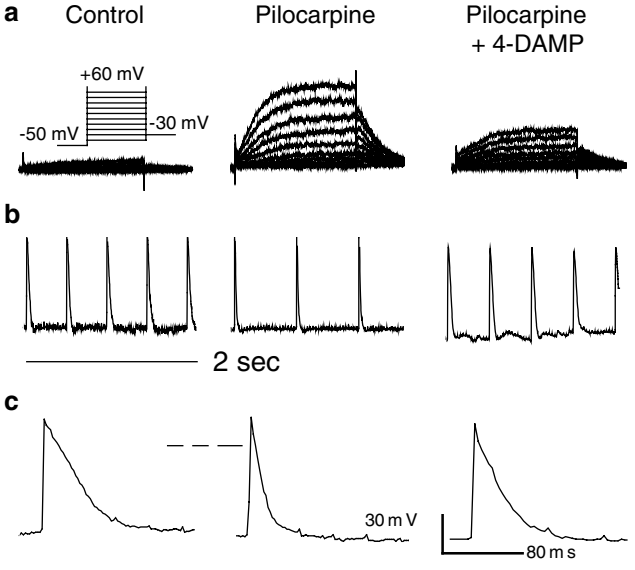

Figure 2 (a) Pilocarpine induction of a delayed rectifier K^+ current *via* stimulation of M_3 receptors (I_{KM3}) in isolated single guinea-pig atrial myocytes. Currents were elicited by 2-s pulses to potentials ranging from −40 to +50 mV with 10-mV increment, followed by a 1-s step to −30 mV. Voltage steps were delivered from a holding potential of −50 mV at an interpulse interval of 5 s. Shown are raw current traces recorded under control conditions (in the presence of M_2 antagonist methoctramine, 100 nM, throughout the experiment), in the presence of pilocarpine (10 μM) and after addition of 4-DAMP (2 nM, an M_3-selective antagonist) to the pilocarpine-containing solution. (b) Pilocarpine modulation of sinus rate *via* stimulation of mAChRs and reversal by 4-DAMP (10 nM) in guinea-pig atria. Sinus rate was determined as the firing frequency of action potentials (AP) recorded in atrial preparations with intact sinus nodes. (c) Pilocarpine modulation of APD by activation of mAChRs and reversal by 4-DAMP in guinea-pig atria. The dash line indicates zero potential level.

Upon exposure of a myocyte to an mAChR receptor agonist, the so-called ACh-activated inward rectifier K^+ current (I_{KACh}) is activated, followed by desensitization, that is, a decay of the current (Bünemann *et al*., 1996). I_{KACh} has been generally thought to be mediated by stimulation of the M_2 receptors. However, some recent studies indicate that the M_3 receptors might also be involved in I_{KACh} activation and desensitization. Primarily based on the pharmacological evidence, Kobrinsky *et al*. (2000) hypothesized that the activation and the fast desensitization of I_{KACh} in rat atrial cells are mediated by the M_2 and the M_3 receptors, respectively; the M_2 subtype causes activation of I_{KACh} *via* $G_{i/o}$, and the M_3 subtype causes desensitization *via* $G_{q/11}$, because 4-DAMP and a PLC inhibitor, the aminosteroid U73122, both prevented the fast desensitization. Another study also showed that 4-DAMP, at 10 nM, caused a reversible reduction of I_{KACh} induced by 2 μM ACh by about 40% (Meyer *et al*., 2001). However, these authors interpreted the data as inhibition of the M_2 receptors by 4-DAMP because they were unable to detect the M_3 mRNA in their samples. Neither were they able to observe any changes in activation of I_{KACh} in cells pretreated with the *P. multocida* toxin that uncouples G_q proteins from their receptors. Similarly, the work published by Cho *et al*. (2002) demonstrated that 50 nM 4-DAMP significantly reduced I_{KACh} peak amplitude and the fast phase of desensitization was nearly abolished in mouse atrial myocytes. Meanwhile, the activation time course of I_{KACh} was markedly slowed by 4-DAMP. Moreover, the M_3 transcript was detected in their preparations. These data support the view that the M_3 receptors contribute to I_{KACh} activation and rapid desensitization.

Evidence for the functional M_3 receptors in the human heart has also been acquired by researchers. Bristow (1993) reported that carbachol at high concentrations increased IP formation. This increase in IP formation could be viewed as a result of stimulation of one of the mAChR subtypes that normally couple to the $G_{q/11}$-PLC pathway, such as the M_3 receptors. Alternatively, it could also be due to stimulation of the M_2 receptors, resulting in activation of the α-subunit of G_i with subsequent release of the $\beta\gamma$-complex, which has been shown to be capable of activating PLC (Wess, 1996). The data in support of the former were documented by a recent study from Brodde's group (Willmy-Matthes *et al*., 2003), which is a continuation of their study in rat hearts (Pönicke *et al*., 2003) as already described above. Their study in human right atrial slices demonstrated that pirenzepine and himbacine, used in concentrations that occupy M_1 and M_2 receptors, respectively, by ~80–100%, did not significantly affect carbachol-induced IP formation. On the other hand, darifenacin concentration-dependently inhibited carbachol-induced IP formation with a pK_i value of 8.5, well in line with its affinity for the M_3 subtype (Caulfield & Birdsall, 1998). The authors contemplated that the well-recognized ability of carbachol to cause positive inotropic effects in human hearts (Dhein *et al*., 2001) is mediated by M_3-receptor stimulation. With a different approach, Dobrev *et al*. (2002) recently studied the role of M_3 receptors in activation of I_{KACh} in human atrial myocytes. As already mentioned, I_{KACh} is generally believed to be activated by M_2 receptors only. However, their data demonstrated that 4-DAMP mustard, at a concentration of 10 nM which is supposed to antagonize mainly the M_1 and M_3 receptors, reduced I_{KACh} by ~30%. They proposed that

mAChRs mediate activation of I_{KACh} in human atrial myocytes not only by M_2 but also by M_1 and M_3 receptors, in agreement with the findings on I_{KACh} in rat and mouse atrial cells as already discussed above. These functional evidences from human hearts are in agreement with our earlier molecular data indicating the presence of the M_3 receptor proteins in the cytoplasmic membrane of human cardiac atrial and ventricular myocytes (to be discussed below) (Wang *et al*., 2001). Furthermore, in line with the finding from Ravens' group (Dobrev *et al*., 2002), we have recently found that, in the atria from dogs with tachypacing-induced congestive heart failure (CHF), the density of the M_3 receptors increased, whereas that of the M_2 receptors decreased (Shi *et al*., 2004). This suggests a possibility of reversed relative contributions of the M_2 and M_3 receptors with the M_2 predominant in physiological conditions and the M_3 more prominent under pathological situations.

Molecular biology evidence

Expression of mRNAs encoding different subtypes of mAChRs ($M_1/M_2/M_3/M_4$) in chick hearts has been confirmed by two groups (Tietje & Nathanson, 1991; Gadbut & Galper, 1994). While the results from these pioneering studies at the molecular level in avian may not be necessarily extrapolated to the mammals, they did initiate subsequent exploring of diversity of mAChRs in mammalian hearts at the molecular level.

Krejči & Tuček (2002) conducted a quantitative study on mRNAs for M_1–M_5 subtypes in rat heart, using RT–PCR. Their data showed expression of the M_3 transcripts in atria and left/right ventricles, albeit at very low levels compared with the M_2 mRNA. The M_3 transcript was also detected in isolated mouse atrial myocytes (Cho *et al*., 2002). More importantly, this same group found that carbachol increased PI hydrolysis in atrial myocytes isolated from wild-type mice, and this increase was substantially decreased (by $\sim$45%) in PLCβ1 knockout mice, indicating a potential role of PLC activation through G_q-coupled mAChRs (e.g. the M_3 receptors) in PI hydrolysis.

Our laboratories have also performed molecular analyses of mAChRs in canine atria. We cloned cDNA fragments of 458 bp for M_2 (accession no. AF056305) and of 432 bp for M_3 (accession no. AF056305) isoforms from canine atrial mRNA preparations. These sequences represent a part of the third intracellular loop between transmembrane domains 5 and 6, which is thought to contain critical determinants of G protein-coupling specificity. These two fragments have little identity with each other, but share 91 and 81% homology to the same regions of corresponding human M_2 and M_3 sequences, respectively, in the amino-acid level. Using these cDNA fragments, we designed primers for RT–PCR detection of M_3 transcripts. Significant expression of the M_3 transcript was consistently identified in canine atrial RNA samples (Shi *et al*., 1999a).

Molecular evidence for the cardiac M_3 receptors has also been obtained from human hearts. Hellgren *et al*. (2000) and Oberhauser *et al*. (2001) demonstrated, independently, the presence of mRNA for the M_2 and M_3 receptors in left/right atria/ventricles of human hearts, although the M_2 mRNA was found to be much more abundant than the M_3 mRNA. Further evidence was reported in our recent study (Wang *et al*., 2001) that revealed the expression of the M_3 gene, and the presence and subcellular localization of the M_3 receptor proteins in the cytoplasmic membrane of human atrial and ventricular cells. mRNAs encoding the M_2 and M_3 receptors were readily detected by RT–PCR in both atrial and ventricular samples. More favorable data for the co-existence of the M_2 and M_3 receptors in human hearts were acquired from the Western blot analyses of membrane proteins with subtype-specific antibodies. The protein levels of the M_2 isoform were comparable between human atrium and ventricle, while the density of M_3 appeared $\sim$10-fold higher in human ventricle than in atrium. Consistent with the Western blot data, cells exposed to antibodies against M_2 or M_3 receptors showed clear sarcolemmal staining. Under confocal microscopy, the M_2 receptors were found to be evenly distributed throughout the surface membrane, whereas the M_3 proteins demonstrated stronger staining on the intercalated discs relative to other regions of the plasma membrane (Figure 3).

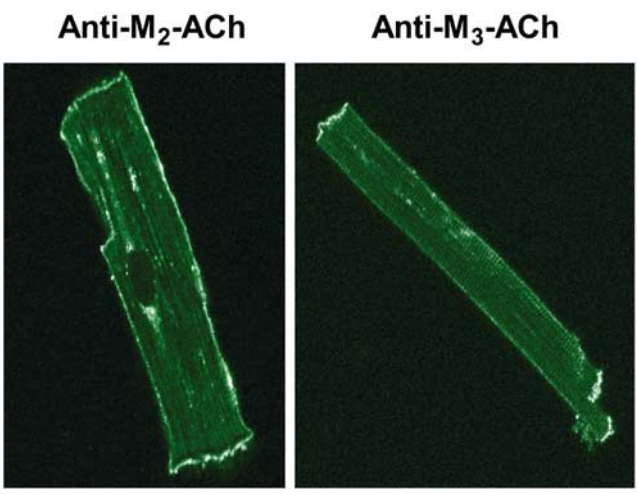

Figure 3 Immunostaining of isolated human ventricular myocytes with antibodies directed against the M_2 and M_3 subtypes of mAChR, respectively. Note that the anti-M_2-ACh antibody stains the cell membrane evenly, whereas the anti-M_3-ACh antibody preferentially stains the intercalated discs relative to other areas of the plasma membrane.

Evidence for homogeneous M_2 receptor expression

There have been surprisingly few systematic pharmacological studies with the use of various subtype-selective antagonists to verify that the heart possesses only the M_2 receptors. Functional studies in this regard have also been scanty. Most of the evidence for 'homogeneous M_2 receptor expression' was acquired from molecular biology studies.

Pharmacological evidence

Several studies employed pure pharmacological approach or radioligand-binding assays with various subtype-selective antagonists generated data supporting the 'homogeneous M_2 receptor expression' concept in the heart. Michel *et al*. (1989) reported that, in the rat submaxillary gland, [^{3}H]4-DAMP predominantly bound with high affinity ($K_d = 0.2$ nM) to a

population of binding sites that displayed pharmacology of the M_3 muscarinic receptor subtype. In rat heart, however, [^{3}H]4-DAMP labeled the M_2 muscarinic receptor with low affinity ($K_d = 4$ nM). Similar results were obtained by the same group in another study (Delmendo *et al*., 1989). Moriya *et al*. (1999) examined the antagonist-binding profiles of nine muscarinic antagonists (atropine, 4-DAMP, pirenzepine, oxybutynin, tiquizium, timepidium, propiverine, darifenacin and zamifenacin) for human mACh subtypes (M_1, M_2, M_3, M_4 and M_5) produced by using a baculovirus infection system in Sf9 insect cells, and rat tissue membrane preparations (heart and submandibular gland). The binding affinities of muscarinic antagonists for rat heart and submandibular gland strongly correlated with those for human cloned M_2 and M_3 subtypes, respectively (Moriya *et al*., 1999), in favor of the cardiac 'homogeneous M_2 receptor expression' theory. The second study characterized the displacement of bound [^{3}H]NMS by atropine, AF-DX 116 and HHSiD in human atrial and ventricular myocardium (Deighton *et al*., 1990). They found that the antagonists inhibited [^{3}H]NMS binding to right atrial and left ventricular membranes, with steep, monophasic competition curves indicating interaction with a single class of binding sites. In both tissues, the order of potency was: atropine > AF-DX 116 > HHSiD > pirenzepine. In agreement with the radioligand-binding experiments, the antagonists antagonized the negative inotropic effect of carbachol in both human atria and ventricles with an order of potency: atropine > AF-DX 116 > HHSiD > pirenzepine. The authors interpreted the data as of favoring 'homogeneous M_2 receptor expression' concept in the human heart. Another binding study declared the presence of only the M_2 receptors in rat hearts (Giraldo *et al*., 1988). However, in this study, only two subtype-selective antagonists pirenzepine and AF-DX 116 were used.

Functional evidence

The parasympathetic control of the heart rate and cardiac contraction force is commonly used as a biomarker of the M_2 function; thus, studies on cholinergic regulation of heart rate and contraction are generally considered as a consequence of M_2 receptor activation. Acetylcholine (ACh) released from the stimulated vagal nerve decreases heart rate *via* modulation of several types of ion channels expressed in cardiac pacemaker cells. It is well established that activation of I_{KACh} induced by ACh or other mAChR agonists in mammalian cardiomyocytes is mediated by the M_2 receptors (Yatani *et al*., 1988; Logothetis *et al*., 1988; Sato *et al*., 1990; Shi *et al*., 1999a; 2003), although some recent studies have suggested a contribution from the M_3 receptors (Meyer *et al*., 2001; Cho *et al*., 2002; Dobrev *et al*., 2002). Moreover, in guinea-pig heart, it has been shown that pre- and post-junctional mAChRs are of the M_2 subtype (Jeck *et al*., 1988). Wickman *et al*. (1998) assessed the role of I_{KACh} in heart rate regulation *in vivo* using a mouse line deficient in I_{KACh} by targeted disruption of the gene coding for GIRK4, one of the channel subunits. They analyzed the heart rate and heart rate variability at rest and after pharmacological manipulation in unrestrained conscious mice using electrocardiogram telemetry, and found that I_{KACh} mediated approximately half of the negative chronotropic effects of vagal stimulation on heart rate. It is assumed that stimulation of mAChRs leads to pacemaker current I_f inhibition *via* a PTX-sensitive G-protein, resulting in inhibition of adenylyl cyclase and reduced cAMP production. This alters I_f availability (DiFrancesco & Tromba, 1988), since this channel is directly cAMP sensitive (DiFrancesco & Tromba, 1987). This effect is believed to participate in the effects of cholinergic attenuation of heart rate. The cardiac L-type Ca^{2+} current (I_{CaL}) is inhibited by muscarinic stimulation in both atrium and ventricle. However, while in atrium no prior elevation of intracellular cAMP concentration is required, in ventricle the inhibitory effect on I_{CaL} is typically seen only if cAMP has been elevated (Mery *et al*., 1997; Imai *et al*., 2001). This effect is thought to account for the negative inotropic regulation by cholinergic stimulation. Muscarinic modulation of both I_f and I_{CaL} depends on reduction of cAMP, indicating the requirement of M_2 receptor stimulation, despite the fact that the role of the M_2 receptors has never been confirmed and the potential involvement of other subtypes has never been excluded. Du *et al*. (2001) performed a study on the inotropic effects of ACh using subtype-selective muscarinic receptor antagonists, pirenzepine, AF-DX 116 and HHSiD, the human myocardium. Their results revealed that the negative inotropic effect of acetylcholine in atrial as well as the positive inotropic effect in ventricular trabeculae were best antagonized by AF-DX 116 and not by pirenzepine, suggesting the involvement of the muscarinic M_2 receptor subtype.

Molecular biology evidence

Hoover *et al*. (1994) reported a study using *in situ* hybridization histochemistry with [^{35}S]-labeled oligonucleotide probes to explore if there is expression of other mACh genes in addition to M_2 mRNA at discrete sites within the rat myocardium and by intrinsic cardiac ganglia. Their results demonstrated expression of mRNAs for multiple subtypes of mAChR (M_1, M_2 and M_4) in the intrinsic cardiac ganglia, but only M_2 mRNA was detected in the myocardium. Similar experiments were also conducted by Hassall *et al*. (1993) in rat and guinea-pig intracardiac neurons and atria, employing both [^{35}S]- and digoxigenin-labeled oligonucleotide probes specific for mRNAs of all the five mAChR subtypes. The authors found that, while all intracardiac neurons expressed mRNAs for M_1–M_4 subtypes, atrial myocytes in culture were only labeled by [^{35}S]- and digoxigenin-tailed M_2 oligonucleotides. With RT–PCR, M_3 transcripts were not detected either, but the M_2 mRNA was found to be expressed at a high level, in rat atrial myocytes (Meyer *et al*., 2001). Earlier than these studies, tissue distribution of the mRNAs encoding M_1–M4 mAChRs has been investigated by blot hybridization analysis with specific probes by Maeda *et al*. (1988). This study showed that exocrine glands contained the mRNAs for the M_1 and M_3 subtypes, whereas smooth muscles contained the mRNAs for the M_2 and M_3 subtypes. All the four mAChR mRNAs were present in the cerebrum, whereas only M_2 mRNA was found in the heart. Similar mRNA expression profile in rats was also reported by Peralta *et al*. (1987) and Franco *et al*. (1997).

Immunoprecipitation assays using muscarinic receptor subtype-specific antisera were used to measure the relative levels of M_1, M_2, M_3 and M_4 receptors at the protein level. The M_2 receptor was found to be the predominant subtype in the bladder and uterus, and the only subtype detected in rabbit heart (Brandes & Ruggieri, 1995).

By using targeted mutagenesis in mouse embryonic stem cells, Gomeza *et al*. (1999) generated mice lacking functional M_2 receptors and demonstrated an obligatory role of the M_2 receptors in regulation of heart rate. In atria from M_2-receptor knockout mice, carbachol had no effect on the beating rate. Similarly, Stengel *et al*. (2000) showed that carbachol produced bradycardia in spontaneously beating atria isolated from M_4-receptor knockout mice not different from wild-type mice, while in atria from M_2 knockout mice, carbachol did not produce bradycardia. The atrial rate was found by the same group (Stengel *et al*., 2002) to be similar in M_3 receptor knockout and wild-type mice. Based on this observation, the authors claimed that the M_3 receptors do not contribute to heart rate control.

Multiple myocardial receptors?

Several issues must be considered when evaluating the previous studies

(1) *Limitations of approaches*: Pharmacological characterization may lead to false interpretation because of imperfect selectivity of mAChR antagonists available. As shown in Table 1, any of the 'subtype-selective' antagonists towards a given mAChR subtype can crossreact with other subtypes. Functional characterization could be misleading too. For example, heart rate control or activation of I_{KACh} has been used as a marker for the presence and function of the cardiac M_2 receptors and for excluding the presence of other subtypes. However, as already described, there is now evidence indicating the participation of the M_3 receptors in heart rate control (Wang *et al*., 1999a; Shi *et al*., 1999b) and I_{KACh} activation (Kobrinsky *et al*., 2000; Meyer *et al*., 2001; Cho *et al*., 2002; Dobrev *et al*., 2002). Similarly, IP formation is used as an indication of the M_3 receptor function (or other 'odd' number mAChRs known to couple to the $G_{q/11}$/PLC pathway). However, IP formation could also be due to stimulation of the M_2-receptors resulting in activation of the α-subunit of G_i with subsequent release of the $\beta\gamma$-complex, which has been shown to be capable of activating PLC (Wess, 1996). Molecular biology studies should provide unequivocal evidence for mAChR subtype expression; yet, this is not always true. While Northern blot is superior for detecting mRNA expressed at sufficiently high levels which likely generate functional protein products, it has an inherent low sensitivity and may not detect low-abundance transcripts. If the level of the M_3 transcript is below the detection threshold of Northern blot analysis, then the expression of M_3 may be overlooked. Well-controlled RT–PCR method with carefully designed gene-specific primers can provide a better tool for detecting low-abundance expression for this technique is some 1000 times more sensitive than Northern blot and can detect very low levels of mRNA expression. However, it also can generate data that are difficult to interpret. First, mRNA samples may be contaminated by the source from noncardiac cells, such as intrinsic cardiac neurons, vascular cells, fibroblasts, etc. Second, the mRNA detected by RT–PCR may not necessarily represent the functional entity, simply because expression at very low abundance may not produce the corresponding protein. Antibodies directed against subtypes of mAChR would be unambiguous for establishing expression and localization of mAChR subtypes down to the cytoplasmic membrane. Unfortunately, the availability of commercial antibodies for mAChR subtypes is rather limited and specificities of the available antibodies remain yet to be fully verified. Alternatively, the mAChR knockout animals would be the most reliable tools for determining the function of mAChR subtypes. This technique has actually been used in M_1–M_5 muscarinic receptor knockout mice to study the physiological roles of the muscarinic cholinergic system (Wess, 2003; Wess *et al*., 2003; Bymaster *et al*., 2003). As already described above, studies with M_2, M_3 or M_4 knockout mice model indicated that only the M_2 receptors are operating in regulating heart rate (Gomeza *et al*., 1999; Stengel *et al*., 2000; 2002). Cautions therefore need to be taken when interpreting the results using any of these approaches and methods.

(2) *Animal species*: Cardiomyocytes from different species may express different genes, say, different subtypes of mAChRs. By far, evidence for the presence of the M_3 receptors has been documented in the hearts of various species including humans, dogs, cats, rabbits, guinea-pigs, rats, mice, and chicks. On the other hand, evidence against the presence of the M_3 receptors in the heart has also been obtained from rats, mice, guinea-pigs and rabbits. Studies concerning mAChR subtypes in human hearts have been sparse and it is noteworthy that existing studies on this subject mostly reported positive evidence for the presence of the M_3 subtype in human myocardium. Similarly, the published studies to date involving mAChR subtypes in canine hearts all reported results in favor of the presence of the M_3 receptors. This would imply that there might be interspecies differences in terms of the mAChR subtypes expressed in the heart.

(3) *Pathological conditions*: Dobrev *et al*. (2002) investigated the role of the M_3 receptors in activation of I_{KACh} in atrial myocytes from patients with sinus rhythm and chronic atrial fibrillation (AF). They found that I_{KACh} was significantly enhanced in AF patients and 4-DAMP mustard (10 nM) inhibited normalized I_{KACh} amplitude to a significantly greater extent in the atrial myocytes from AF patients ($\sim$62% reduction) than in those from healthy patients with sinus rhythm ($\sim$30% reduction). They consider these results as an indication of increased contribution of the M_1 and M_3 receptors to I_{KACh} activation in AF patients. This result warns us that the proportion of expression and the relative contributions to the cardiac function, from different subtypes of mAChR, might alter with pathological situations. Our laboratories have recently obtained data in support of this notion. We compared the M_2 and M_3 receptors densities by Western blotting analysis, and I_{KACh} and I_{KM3} currents density by whole-cell patch-clamp techniques, in atrial myocytes isolated from dogs with congestive heart failure (CHF) induced by tachypacing (Shi *et al*., 2004). We found that the density of M_2 receptors was significantly reduced, accompanied by decreased I_{KACh} current density in atrial cells from the dogs with CHF relative to the control healthy dogs. In sharp contrast, M_3 receptor density was remarkably increased and, correspondingly, I_{KM3} current density was also increased in CHF dogs (Figure 4). These facts suggest that pathological conditions are another factor influencing the expression and relative contribution of the cardiac M_3 receptors; a minor role of the M_3 receptors under physiological conditions might become prominent under pathological situations.

From the above discussion, we can safely conclude that, under physiological conditions, the M_2 subtype is the

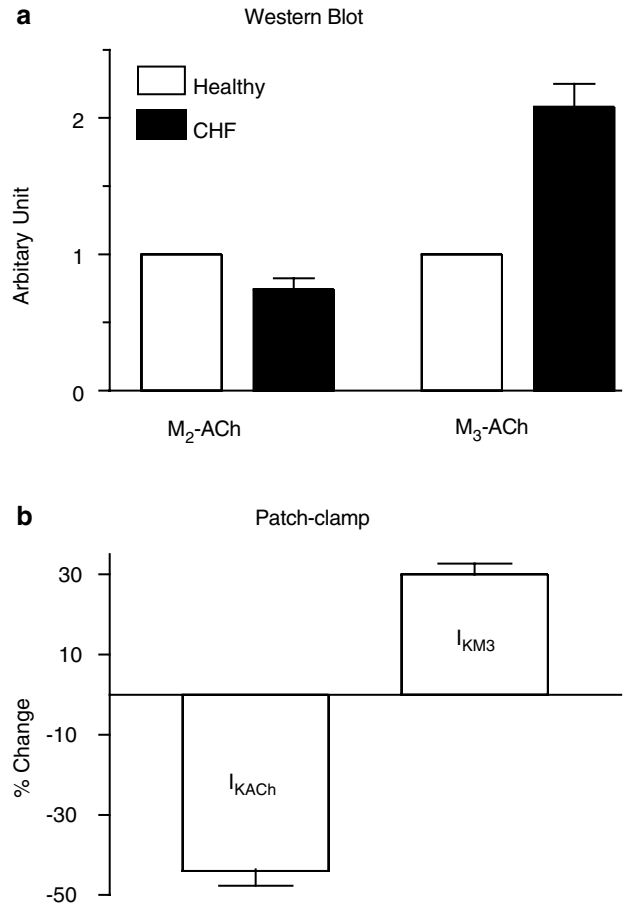

Figure 4 Comparisons of the protein levels between M_2 and M_3 (a) and of the current densities between I_{KACh} and I_{KM3} (b), in protein samples or myocytes isolated from right atria of healthy dogs ($n=4$) and the dogs with congestive heart failure (CHF, $n=4$) induced by rapid ventricular pacing. Note the decreased level of M_2 receptors ($P<0.05$) accompanied by the decreased I_{KACh} ($P<0.01$), and increased the level of M_3 receptors ($P<0.01$) with increased I_{KM3} ($P<0.05$), in CHF dogs as compared with the healthy dogs.

predominant mAChR over the M_3 and other subtypes being expressed and functioning in the heart, and it is the major subtype responsible for the chronotropic and inotropic regulations by the parasympathetic control. Nonetheless, the role of other subtypes, particularly the M_3 subtype, cannot be overlooked.

Implications for myocardial receptor heterogeneity

mAChRs are found in various cardiac tissues including sinus node, atrium, A–V node and ventricle (Siegel & Fischbach, 1984; Sorota *et al*., 1986; Giessler *et al*., 1999; Hellgren *et al*., 2000). mAChRs play an important role in mediating parasympathetic effects on the heart function. The principal effects of mAChR stimulation in the heart are (1) slowing or accelerating the heart rate (negative or positive chronotropic effects), (2) weakening or strengthening the contractile force (negative or positive inotropic effects), (3) shortening the atrial APD, (4) attenuating the atrioventricular nodal conduction velocity and (5) reducing the cardiomyocyte apoptotic cell death.

The general finding is that low concentrations of muscarinic agonists cause decreases in heart rate, atrioventricular conduction and ventricular contraction. Paradoxically, under appropriate conditions, activation of cardiac mAChRs elicits stimulatory effects on the rate of beating and contractile force of the heart. These latter effects often require higher concentrations of agonists and in some cardiac cell types are only seen after pretreatment with pertussis toxin (PTX), indicating the involvement of non-M_2 subtypes (Löffelholz & Pappano, 1985). The dual effects of muscarinic agonists cannot be readily explained by the functional profile of the M_2 receptors.

Cholinergic activity plays an important role in superaventricular arrhythmias and activation of I_{KACh}, which is mediated by $\beta\gamma$-subunits of G_i protein (Reuveny *et al*., 1994; Wickman & Clapham, 1995), contributes to initiation and perpetuation of AF because the efflux of K^+ through I_{KACh} tremendously accelerates cardiac repolarization and shortens the effective refractory period, an effect favoring the occurrence of re-entrant arrhythmias. Although I_{KACh} is commonly believed to be induced by activation of the M_2 receptors, recent studies have demonstrated potential participation of the M_3 subtype (Meyer *et al*., 2001; Cho *et al*., 2002; Dobrev *et al*., 2002) and a role of the M_3 receptors in I_{KACh} activation was found to be increased in AF. These results at least indicate a pathological role of the M_3 receptors in initiating and maintaining AF. This notion is further supported by our finding that stimulation of the M_3 receptors activates I_{KM3} in canine and guinea-pig atria. I_{KM3} may also contribute to AF. Indeed, it is known that dogs with tachypacing-induced congestive heart failure (CHF) are more prone to AF initiation and perpetuation. We have found that, in the atria of dogs with CHF, the densities of the M_3 receptors and I_{KM3} current are both robustly increased (Figure 4), but those of the M_2 are abrogated (Shi *et al*., 2004). The pacemaker current I_f plays a critical role in spontaneous beating of the heart and cholinergic stimulation decreases the heart rate partly by inhibiting I_f (DiFrancesco & Tromba, 1987; 1988; Yatani *et al*., 1990). The mechanisms of I_f suppression by ACh involve inhibition of basal adenylate-cyclase activity (DiFrancesco & Tromba, 1987; 1988) or/and direct coupling to α-subunit of G_o protein within the plasma membrane (Yatani *et al*., 1990), therefore the effect is mostly likely mediated by the M_2 receptors. Whether the M_3 receptors also participate in I_f regulation is unknown.

The data in the literature regarding changes of mAChRs density in the subjects with cardiomyopathy have been controversial. Some studies corroborate the idea that the number or the function of cardiac mAChRs is not changed in cardiomyopathic patients (Böhm *et al*., 1990; Brodde *et al*., 1992; Fu *et al*., 1992). On the other hand, *in vivo* positron emission tomography (PET) using [^{11}C]methylquinuclidinyl benzilate as ligand demonstrated slightly enhanced mAChR density in patients with CHF *vs* healthy controls (Le Guludec *et al*., 1997). In addition, Koumi *et al*. (1994) described reduced I_{KACh} channel sensitivity to M_2 receptor-linked G_i protein in the atrial cells from patients suffering from chronic heart failure as compared to the atrial cells from non-failing hearts. In the rats with aortic banding and substantial cardiac hypertrophy, both mAChR density and functional responsive-

ness dropped (Mertens *et al*., 1995). In a similar manner, Vatner *et al*. (1988) found dwindled density and functional responsiveness of cardiac mAChRs, but unchanged cardiac G_i protein, in an aortic banding dog model of cardiac failure. Diminished expression of cardiac mAChR has also been described in a rat model of chronic heart failure induced by ethanol (Strasser *et al*., 1996). One possible explanation for the disparities among different studies is that the total density of mAChRs may or may not alter depending on proportional changes of different subtypes under a particular pathological condition. For example, the density of the M_2 receptors falls but that of the M_3 subtype increases in the atria of experimental CHF (Figure 4) (Shi *et al*., 2004).

The M_3 receptors may thus play a role in parasympathetic control of heart function under normal physiological conditions and in some pathological processes. However, to finally resolve this issue, superior pharmacological tools are required, along with further studies in transgenic animals.

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