Muscarinic Receptor Agonists and Antagonists: Effects on Cardiovascular Function

Robert D. Harvey

Abstract Muscarinic receptor activation plays an essential role in parasympathetic regulation of cardiovascular function. The primary effect of parasympathetic stimulation is to decrease cardiac output by inhibiting heart rate. However, pharmacologically, muscarinic agonists are actually capable of producing both inhibitory and stimulatory effects on the heart as well as vasculature. This reflects the fact that muscarinic receptors are expressed throughout the cardiovascular system, even though they are not always involved in mediating parasympathetic responses. In the heart, in addition to regulating heart rate by altering the electrical activity of the sinoatrial node, activation of M_2 receptors can affect conduction of electrical impulses through the atrioventricular node. These same receptors can also regulate the electrical and mechanical activity of the atria and ventricles. In the vasculature, activation of M_3 and M_5 receptors in epithelial cells can cause vasorelaxation, while activation of M_1 or M_3 receptors in vascular smooth muscle cells can cause vasoconstriction in the absence of endothelium. This review focuses on our current understanding of the signaling mechanisms involved in mediating these responses.

Keywords Blood vessels • Cardiac muscle • Heart • Vascular endothelium • Vascular smooth muscle

1 Introduction

The parasympathetic branch of the autonomic nervous system plays an integral role in regulating the cardiovascular system. In general, parasympathetic stimulation tends to produce responses that counterbalance those that are associated with

R.D. Harvey (✉)
Department of Pharmacology, University of Nevada School of Medicine, Reno, NV 89557, USA
e-mail: rdharvey@medicine.nevada.edu

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activation of the sympathetic nervous system (Levy 1971; Löffelholz and Pappano 1985). The tightly orchestrated interactions between sympathetic and parasympathetic responses are essential to maintaining homeostasis of the cardiovascular system under a variety of conditions.

Sympathetic stimulation increases cardiac output by increasing heart rate and contractility through the effects of the neurotransmitter norepinephrine acting on cardiac beta-adrenergic receptors (Bers 2001). Sympathetic stimulation also increases vascular resistance by stimulating vasoconstriction through the effects of norepinephrine acting on alpha-adrenergic receptors in vascular smooth muscle cells (Hirst and Edwards 1989). The primary effect associated with parasympathetic stimulation, on the other hand, is to decrease cardiac output by decreasing heart rate through the effects of the neurotransmitter acetylcholine (ACh) acting on muscarinic receptors (Hartzell 1988; Levy 1971; Löffelholz and Pappano 1985). Under most conditions, parasympathetic stimulation has little effect on cardiac (ventricular) contractility (Levy and Martin 1989). Furthermore, parasympathetic stimulation exerts limited influence on most blood vessels and is not a major factor in regulating total peripheral resistance (Eglen and Whiting 1990; Furchgott and Vanhoutte 1989).

Based on the simplified generalizations just described, the perception is often that muscarinic signaling pathways play an important, yet perhaps more limited physiologic role in regulating cardiovascular function. Yet this notion belies the fact that muscarinic receptors are abundant throughout the cardiovascular system (Eglen and Whiting 1990; Löffelholz and Pappano 1985). As a result, muscarinic agonists as well as antagonists can have profound pharmacologic effects on many aspects of cardiovascular function not normally thought to be under significant parasympathetic influence. For example, muscarinic receptor agonists can actually produce a significant decrease in ventricular contractility in the presence of elevated sympathetic tone (Levy 1977, 1995). Likewise, muscarinic receptor agonists can cause vasodilation of most blood vessels, resulting in a decrease in total peripheral resistance (Furchgott and Zawadzki 1980). These observations, and others, illustrate the more complex nature of the role that muscarinic responses may play in regulating cardiovascular function in health and disease.

2 Cardiovascular Muscarinic Receptors

Five muscarinic receptor subtypes have been identified: M_1, M_2, M_3, M_4, and M_5 (Hulme et al. 1990). In the heart, pharmacologic evidence indicates that most functional responses are associated with activation of M_2 receptors (Harvey and Belevych 2003). This is supported by the inability of ACh to produce bradycardia in mice where expression of the M_2 receptor has been knocked out (Stengel et al. 2000). On the other hand, M_3 receptors appear to play a dominant role in ACh-induced vasodilation of most blood vessels (Beny et al. 2008; Khurana et al. 2004). It should be noted that these are broad generalizations, and that other muscarinic

receptor subtypes have been reported to produce effects in different cell types throughout the cardiovascular system that may or may not be involved in the responses described above. There are also some species-dependent differences in the subtype of receptor associated with different responses (Dhein et al. 2001; Eglen and Whiting 1990).

In general, the signaling pathways most often associated with even-numbered muscarinic receptors involve the heterotrimeric G protein G_i coupled to the inhibition of adenylyl cyclase or the regulation of G protein activated inward rectifying K^+ (GIRK) channels (Lanzafame et al. 2003). Whereas the signaling pathway commonly associated with odd-numbered muscarinic receptors involves G_q activation of phospholipase C (PLC) and subsequent production of diacylglycerol (DAG) and inositoltriphosphate (IP_3) (Lanzafame et al. 2003). While these generalizations explain many of the responses that are mediated by muscarinic receptors in the heart and vasculature, there is evidence that additional signaling mechanisms are important as well.

3 Cardiac Muscarinic Responses

Activation of M_2 muscarinic receptors decreases heart rate by slowing the rate of spontaneous action potential firing in the sinoatrial (SA) node (Irisawa et al. 1993). However, muscarinic agonists can produce significant changes in electrical as well as mechanical function of myocytes throughout all regions of the heart. In the atrioventricular (AV) node, muscarinic stimulation slows the conduction of electrical impulses (Martin 1977). This effect plays a critical role in regulating the propagation of action potentials between the atria and ventricles. The dominant effect that parasympathetic stimulation has on the SA and AV nodes parallels the fact that myocytes that make up the SA and AV node have a greater density of muscarinic receptors and are more heavily innervated by the parasympathetic nervous system than myocytes in other regions of the heart (Löffelholz and Pappano 1985).

Under normal resting conditions, the heart receives significant input from the parasympathetic nervous system. The consequence is that tonic muscarinic receptor activation actually inhibits the intrinsic rate of firing of pacemaker cells and slows heart rate (Levy 1977). The tonic influence of the parasympathetic nervous system also slows AV conduction (Martin 1977). Pharmacologically this is important because muscarinic receptor antagonists such as atropine can increase intrinsic heart rate and facilitate AV conduction. On the other hand, resting sympathetic tone has a less pronounced effect on the heart. This contributes to the misconception that muscarinic receptor stimulation plays little role in regulating ventricular function (see below) (Levy 1995).

The principal effects of parasympathetic stimulation often reflect changes in SA and AV node function. Nevertheless, there is also significant parasympathetic innervation of the atria as well as the ventricles (Standish et al. 1994, 1995), and

muscarinic receptors are expressed throughout all areas of the heart, including the ventricular myocardium (Löffelholz and Pappano 1985). In atrial cells, the primary effect of muscarinic stimulation is a decrease in action potential duration. In ventricular tissue, muscarinic receptor activation has little effect unless it occurs in the presence of concurrent β-adrenergic receptor activation. The primary effect of β-adrenergic stimulation on ventricular function is to increase contractility and stroke volume. Therefore, in the presence of β-adrenergic stimulation, M_2 muscarinic receptor activation can have a significant inhibitory effect on ventricular contractility.

Autonomic responses involved in producing changes in cardiac output, such as those associated with baroreceptor reflexes, are often thought of doing so by altering sympathetic and parasympathetic tone in a reciprocal fashion. For example, the normal autonomic response to an increase in blood pressure detected by arterial baroreceptors would be to decrease sympathetic tone, while at the same time increasing parasympathetic tone. Under those circumstances, parasympathetic activation of muscarinic receptors would be expected to decrease heart rate, while having little or no effect on ventricular contractility. However, there are situations where both sympathetic and parasympathetic activity to the heart change in parallel (Paton et al. 2005). For example, hypoxic chemoreceptor responses (Koizumi et al. 1982) and conditions such as sleep apnea (Leung 2009) are associated with increases in both sympathetic and parasympathetic tone. Under such circumstances, parasympathetic stimulation and muscarinic receptor activation would be expected to have a significant effect on ventricular function.

Another common misconception is that muscarinic receptors in the cardiovascular system are always associated with inhibitory responses. The fact is they are linked to stimulatory effects as well (Dhein et al. 2001; Harvey and Belevych 2003). Perhaps most prominent example in the heart is the rebound stimulatory response observed upon termination of muscarinic receptor activation. This type of stimulatory effect reflects the fact that M_2 receptors simultaneously activate inhibitory and stimulatory signaling pathways. The inhibitory effect tends to dominate the stimulatory response in the presence of muscarinic receptor activation. However, the kinetics of the two responses are distinctly different. The inhibitory effect turns on and off rapidly while the stimulatory response turns on and off much more slowly. This type of rebound stimulatory response has been described in both atrial and ventricular myocytes, and it is believed to be responsible for rebound increases in heart rate and contractility observed during transient changes in vagal stimulation (Harvey and Belevych 2003).

3.1 Muscarinic Receptor Activation of GIRK Channels

One of the primary effects that muscarinic stimulation has on cardiac function is a slowing of the heart rate. This response is due to activation of M_2 receptors in the SA node, and a subsequent decrease in the firing rate of the spontaneous, slow

response action potentials that are characteristic of the cells that make up this region of the heart (Irisawa et al. 1993). Activation of M_2 receptors in the SA node results in a hyperpolarization of the maximum diastolic potential as well as a slowing of the rate of spontaneous depolarization. Both of these effects may contribute to a decrease in the overall rate of firing by increasing the time it takes the membrane potential to reach threshold and fire an action potential.

Hyperpolarization of the maximum diastolic potential produced by muscarinic receptor activation is due to an increase in the open probability of GIRK channels (Sakmann et al. 1983). These are the ion channels that generate the ACh-activated K^+ current ($I_{K(ACh)}$) found in SA nodal cells, atrial cells, AV nodal cells, as well as ventricular myocytes of some species. The GIRK channel family consists of four members: GIRK1, GIRK2, GIRK3, and GIRK4. In the heart, $I_{K(ACh)}$ is generated by a heterotetrameric channel consisting of GIRK1 and GIRK4 (Krapivinsky et al. 1995). The actual functional role of these channels varies, depending on the cell type in which they are found. However, much of what we know about the molecular basis for regulation of these channels actually comes from work conducted using atrial myocytes.

Evidence as to actual mechanism linking M_2 receptor activation to changes in channel activity was demonstrated by a series of elegant experiments by Soejima and Noma (1984). They found that the open probability of these channels was only affected when ACh was able to activate receptors in close proximity to the channel. This suggested that the signaling mechanism does not involve a diffusible second messenger. The idea that receptor activation of $I_{K(ACh)}$ involves a G protein-dependent mechanism came from studies demonstrating the requirement for intracellular GTP in order to activate the current (Kurachi et al. 1986a, b). The fact that receptor activation of $I_{K(ACh)}$ could also be blocked by pertussis-toxin (PTX) indicated that the G protein involved was either G_i or G_o (Kurachi et al. 1986a; Pfaffinger et al. 1985). These observations ultimately led to the idea that the receptor and channel are coupled by a membrane-delimited mechanism, whereby the channel was activated by direct interaction with the G protein. The question then became whether or not the channel was being regulated by the α or $\beta\gamma$ subunits of the activated G protein. Although studies were published supporting both possibilities, it is now generally accepted that activation of $I_{K(ACh)}$ in cardiac myocytes involves the direct interaction of the channel with the $\beta\gamma$ subunits of G_i (see Fig. 1) (Kurachi 1995).

In atrial myocytes, muscarinic activation of $I_{K(ACh)}$ plays a much different role in regulation of cellular function. Atria are actually made up of an inhomogeneous population of cells with varying properties. Some cells exhibit spontaneous electrical activity, while others are quiescent, but they all have a diastolic membrane potential that is typically much more negative than that found in SA nodal cells. As such, the effect that muscarinic activation of $I_{K(ACh)}$ has on the diastolic membrane potential is not as pronounced. Instead, the most significant effect that activation of these channels has on atrial cells is a reduction in action potential duration (Ten Eick et al. 1976). Because $I_{K(ACh)}$ channels are weak inward rectifiers, they can contribute significantly to the conductance of the membrane during the plateau of

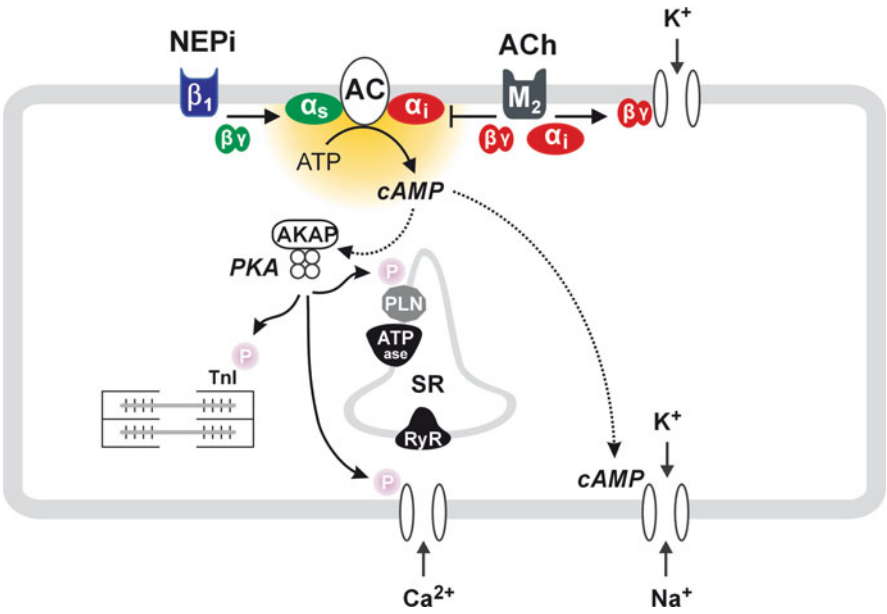

Fig. 1 Muscarinic signaling pathways in supraventricular (sinoatrial, atrial, and atrioventricular) myocytes. Acetylcholine (ACh) acts through M_2 receptors to regulate ACh-activated K^+ channels via a membrane-delimited mechanism involving direct activation by the βγ subunits of the inhibitory G protein G_i. ACh also acts through M_2 receptors to inhibit adenylyl cyclase (AC) activity via the α subunit (α_i) of G_i, resulting in a decrease in cAMP production. This may occur in the absence or presence of agonists that stimulate cAMP production. Norepinephrine (NEPi) acts through β_1-adrenergic receptors to stimulate cAMP synthesis by directly activating all isoforms of adenylyl cyclase (AC) via the α subunit (α_s) of the stimulatory G protein G_s. Changes in cAMP affect targets of protein kinase A (PKA)-dependent phosphorylation such as tropinin I (TnI), phospholamban (PLN), and the L-type Ca^{2+} channel. Changes in cAMP also directly regulate pacemaker channels, which are permeable to both Na^+ and K^+

the action potential, facilitating repolarization. The decrease in action potential duration may also be explained in part by a reduction in cAMP-dependent regulation of the L-type Ca^{2+} current (see below). As a result of the decrease in action potential duration, there is also a decrease in the effective refractory period. This renders these cells more susceptible to excitation by a premature stimulus. This may increase the susceptibility of the atria to arrhythmias (Kovoor et al. 2001). In fact, inhibiting the activation of these channels has been suggested as a treatment for atrial fibrillation (Hashimoto et al. 2006).

Muscarinic activation of $I_{K(ACh)}$ in the AV node plays an important role in regulating action potential propagation. Under normal conditions, the AV node is the only pathway for impulses that originate in the SA node and pass through the atria to reach the ventricles. As such, the AV node plays an essential role in regulating the propagation of impulses from the atria to the ventricles. Activation of muscarinic receptors in the AV node produces a negative dromotropic effect,

or a slowing of impulse propagation. Activation of $I_{K(ACh)}$ may contribute a slowing of conduction by reducing the excitability of AV nodal cells. Reduction in the cAMP-dependent regulation of the L-type Ca^{2+} current may contribute to this effect as well (Nishimura et al. 1988).

Acetylcholine-activated K^+ channels have also been identified in ventricular myocytes of certain species, including frog, ferret, rat, and human (Endoh 1999). However, in those species in which these channels are present in ventricular myocytes, $I_{K(ACh)}$ density is significantly less than that of atrial myocytes. Furthermore, at least in human ventricular myocytes, the channels appear to be much less sensitive to activation by ACh than they are in atrial cells (Koumi and Wasserstrom 1994).

3.2 Muscarinic Regulation of cAMP-Dependent Responses

The other important signaling pathway associated with muscarinic receptor activation in the heart involves modulation of cAMP-dependent responses. As indicated above, the effects of parasympathetic stimulation oppose many of the actions associated with sympathetic stimulation, and sympathetic stimulation exerts many of its acute effects in the heart through β-adrenergic receptor-dependent activation of adenylyl cyclase and subsequent production of cAMP. This pathway modulates a number of key proteins involved in regulating the electrical and mechanical activity of cardiac myocytes (Bers 2002). M_2 muscarinic receptor stimulation can modulate these cAMP-dependent responses through one or more indirect signaling pathways (Harvey and Belevych 2003).

3.2.1 Muscarinic Inhibition of cAMP-Dependent Responses

The dominant effect that M_2 receptor activation has on cAMP-dependent responses is inhibitory and is referred to as "accentuated antagonism" (Levy 1971). This term reflects the fact that the inhibitory response is more prominent in the presence of elevated sympathetic tone. It has been suggested that this type of inhibitory response involves both indirect and direct actions of ACh. The indirect mechanism involves activation of muscarinic receptors on postganglionic sympathetic nerve terminals, which inhibits the release of norepinephrine, preventing subsequent activation of cardiac β-adrenergic receptors. However, muscarinic receptor activation can inhibit responses mediated by β-adrenergic receptor stimulation in isolated myocytes. This demonstrates cAMP-dependent responses can be inhibited by direct activation of cardiac M_2 receptors.

Multiple mechanisms have been suggested to explain how M_2 receptor activation antagonizes cAMP-dependent responses. Perhaps the most widely accepted explanation is based on studies demonstrating that exposure to ACh can reduce cAMP levels in cardiac tissue (Hartzell 1988; Löffelholz and Pappano 1985). This

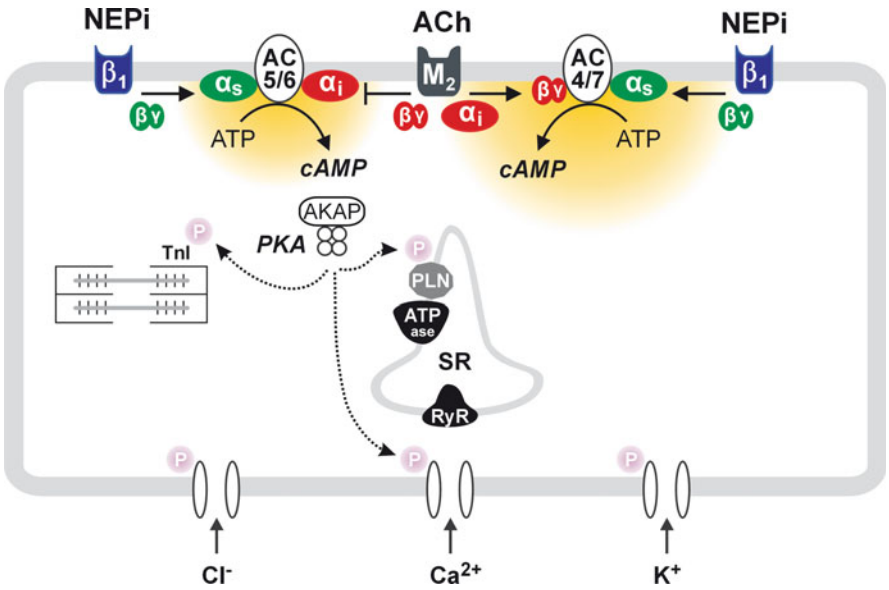

Fig. 2 Muscarinic signaling pathways in ventricular myocytes. Responses to M_2 receptor activation are only observed in the presence of agonists that stimulate cAMP production. Norepinephrine (NEPi) acts through β_1-adrenergic receptors to stimulate cAMP synthesis by directly activating all isoforms of adenylyl cyclase (AC) via the α subunit (α_s) of the stimulatory G protein G_s. Acetylcholine (ACh) acts through M_2 receptors to inhibit AC5/6 activity via the α subunit (α_i) of the inhibitory G protein G_i. ACh acting through M_2 receptors can also stimulate AC4/7 activity via the βγ subunits of G_i. Changes in cAMP affect targets of protein kinase A (PKA)-dependent phosphorylation such as tropinin I (TnI), phospholamban (PLN), as well as L-type Ca^{2+}, delayed rectifier K^+, and CFTR Cl^- channels

effect is due to inhibition of AC activity by a mechanism involving a PTX-sensitive G protein (Endoh et al. 1985). Subsequent biochemical studies have demonstrated that two isoforms of AC expressed in cardiac muscle (AC5 and AC6) can be inhibited by direct interaction with the activated α subunit of the PTX-sensitive G proteins, G_i and G_o (Sunahara et al. 1996). This supports the idea that ACh can antagonize β-adrenergic responses by inhibiting cAMP synthesis (see Fig. 2).

Early studies also demonstrated that exposure to ACh is associated with the production of cGMP in cardiac tissue (George et al. 1970, 1972; Watanabe and Besch 1975). It has been proposed that M_2 receptors stimulate cGMP synthesis through the regulation of endothelial nitric oxide synthase (eNOS) and subsequent production of nitric oxide (NO), which then activates soluble guanylyl cyclase. Furthermore, exogenous cGMP has been reported to inhibit cAMP-dependent responses by activating protein kinase G (PKG) or stimulating type 2 phosphodiesterase activity (Harvey and Belevych 2003; Méry et al. 1997). However, correlations between the effects of ACh and cGMP production have been inconsistent (Hartzell 1988; Löffelholz and Pappano 1985). Furthermore, most studies have found that M_2 receptor antagonism of cAMP response are intact in cardiac

myocytes isolated from the hearts of adult mice in which there has been targeted disruption of eNOS (Belevych and Harvey 2000; Gödecke et al. 2001; Vandecasteele et al. 1999).

Other studies have suggested that the inhibitory effects of ACh do not always correlate with changes in cAMP levels (Hartzell 1988; Lindemann and Watanabe 1989). This has led some to conclude that ACh might antagonize cAMP-dependent responses by stimulating phosphatase activity and enhancing dephosphorylation of proteins phosphorylated by PKA (Ahmad et al. 1989; Gupta et al. 1994). Although such a mechanism could contribute at least partially to the ability of ACh to antagonize cAMP-dependent responses, it has not been possible to demonstrate that ACh directly stimulates the rate of protein dephosphorylation in cardiac myocytes (Stemmer et al. 2000). Furthermore, this mechanism cannot explain the ability of M_2 receptor activation to antagonize responses that do not depend on PKA-dependent phosphorylation, such as direct cAMP-dependent regulation of pacemaker channels (DiFrancesco and Tortora 1991). Dissociation of responses to ACh and changes in cAMP levels may reflect the fact that muscarinic receptor activation appears to affect cAMP production in localized subcellular domains that may be difficult to detect depending on the methods used (Hartzell 1988; Iancu et al. 2007). More recent studies have clearly demonstrated that muscarinic receptor activation causes changes in cAMP activity that can be directly observed in intact, isolated cardiac myocytes using newly developed biosensors (Iancu et al. 2008; Warrier et al. 2005).

The functional consequence of M_2 receptor inhibition of cAMP production varies depending on the cell type involved. In the SA node, muscarinic inhibition of cAMP production contributes to the decrease in heart rate by reversing the effect that cAMP has on the pacemaker channels (DiFrancesco 2010). These channels are regulated by a PKA-independent mechanism that involves direct interaction with cAMP (see Fig. 1). Binding of cAMP shifts the voltage dependence of these channels in a depolarizing direction. This increases their contribution to spontaneous depolarization of the membrane potential during diastole. Muscarinic receptor activation reverses this effect by decreasing cAMP production. This results in a hyperpolarizing shift in the voltage dependence of the channels, reducing their contribution to the rate of spontaneous depolarization. The result is an increase in the amount of time it takes the membrane potential to reach threshold and fire an action potential. The relative importance that activation of $I_{K(ACh)}$ (see above) and inhibition of the pacemaker current play in muscarinic regulation of changes in SA node firing rate and heart rate appear to be concentration dependent. It has been reported that the concentrations of ACh that inhibit the pacemaker current are lower than those required to activate $I_{K(ACh)}$. Muscarinic inhibition of cAMP can also affect the beating rate of SA nodal cells by altering PKA-dependent responses. These include reducing the stimulatory effect that PKA has on L-type Ca^{2+} channel activity (Irisawa et al. 1993). It has also been suggested that inhibition of PKA-dependent regulation of the ryanodine receptor plays an important role in muscarinic inhibition of SA node firing rate, by reducing Ca^{2+} cycling events that

contribute to spontaneous depolarization of the diastolic membrane potential in these cells (Lyashkov et al. 2009).

In atrial myocytes, muscarinic receptor activation can produce a negative inotropic effect (Ten Eick et al. 1976). Part of the inhibitory effect on contractility may be explained by a decrease in cAMP production. The cAMP signaling pathway enhances cardiac myocyte contractility by regulating PKA-dependent phosphorylation of several key proteins. These include, but are not limited to, the L-type Ca^{2+} channel, phospholamban, and troponin I (see Fig. 1). In atrial cells, muscarinic agonists can inhibit contractility by decreasing cAMP production and reversing the actions of PKA-dependent phosphorylation. Some of the inhibitory effect that muscarinic stimulation has on atrial contractility may also be explained by a change in action potential duration that is caused by activation of $I_{K(ACh)}$. Activation of this current contributes to a decrease in action potential duration, which can limit the amount of time available for influx of Ca^{2+} through L-type Ca^{2+} channels, reducing the amplitude of the Ca^{2+} transient.

Muscarinic stimulation can also decrease contractility in ventricular myocytes by inhibiting cAMP production and reversing the effects of PKA-dependent phosphorylation (see Fig. 2). However, ventricular myocyte contractility is not normally influenced of cAMP/PKA-dependent regulation under basal conditions. Therefore, muscarinic inhibition of such responses typically requires prior elevation of cAMP levels through some mechanism that involves increasing adenylyl cyclase activity, such as β-adrenergic receptor stimulation. This type of indirect inhibitory effect is referred to as accentuated antagonism (Levy 1971).

Activation of $I_{K(ACh)}$ does not affect contractility of ventricular myocytes because this current does not contribute significantly to the regulation of membrane potential in most species. On the other hand, muscarinic inhibition of cAMP production does have a significant effect on several channels that do play an important role in regulating the electrical activity of ventricular myocytes. Altering L-type Ca^{2+} channel activity plays an important role in the regulation of cardiac myocyte contractility. However, in addition to affecting contractility, if left unchecked, it would significantly alter action potential duration. Such an effect is potentially arrhythmogenic. To minimize changes in action potential duration, the cAMP/PKA signaling pathway also regulates the activity of ion channels that contribute to repolarization. Depending on the species, these may include delayed rectifier K^+ channels and/or CFTR Cl^- channels (see Fig. 2). Muscarinic receptor stimulation antagonizes the effects that cAMP and PKA have on all of these channels (Hartzell 1988; Harvey and Belevych 2003).

Accentuated antagonism is particularly evident when it comes to explaining the effects of parasympathetic stimulation on ventricular function. In most mammals, muscarinic responses are only observed in adult ventricular myocytes under conditions where cAMP production has been enhanced above basal levels (Hartzell 1988; Harvey and Belevych 2003). This is in contrast to atrial and sinoatrial node cells, where M_2 receptor activation can produce changes in ion channel function typically associated with antagonism of cAMP-dependent responses even in the absence of an agonist that stimulates cAMP production (Dhein et al. 2001; Harvey

and Belevych 2003). This is consistent with the idea that even under basal conditions these cells exhibit a higher basal level of cAMP, which can then be inhibited by muscarinic receptor activation (Méry et al. 1997).

3.2.2 Muscarinic Facilitation of cAMP-Dependent Responses

Despite the fact that M_2 receptor activation can inhibit cAMP-dependent responses, the same receptor acting through the same inhibitory G protein can also produce significant stimulatory effects that are due to facilitation of cAMP production. While both are activated simultaneously, the inhibitory effect dominates. However, upon termination of M_2 receptor activation, the inhibitory effect turns off rapidly, revealing the stimulatory effect, which turns off more slowly. One clear manifestation of such effects is the rebound increase in heart rate and ventricular contractility that can be observed immediately following termination of vagal stimulation or exposure to ACh (Harvey and Belevych 2003).

In atrial myocytes, ACh-induced rebound responses are blocked by inhibition of calmodulin, constitutive NOS activity, soluble guanylyl cyclase, and type 3 phosphodiesterase (PDE3) activity (Wang et al. 1998). This supports the conclusion that ACh-induced rebound stimulation of atrial responses is mediated by Ca^{2+}-calmodulin-dependent activation of NOS, NO-dependent stimulation of soluble guanylyl cyclase, and cGMP-dependent inhibition of PDE3. The result is a decrease in cAMP degradation and facilitation of cAMP-dependent responses. The rebound stimulatory response associated with termination of muscarinic receptor activation has been shown to affect cAMP-dependent regulation of the L-type Ca^{2+} current in atrial myocytes as well as the pacemaker current in SA nodal cells. It has been proposed that this type of response explains the rebound increase in heart rate observed upon termination of vagal stimulation (Wang and Lipsius 1996).

Despite evidence that the NO/cGMP signaling pathway is involved in mediating muscarinic stimulatory responses in atrial myocytes, this is not the case in ventricular myocytes. Rebound stimulatory responses are not blocked by inhibiting this signaling pathway in ventricular cells (Belevych et al. 2001; Zakharov and Harvey 1997). Furthermore, muscarinic stimulatory responses are intact in myocytes isolated from NOS3-KO mice (Belevych and Harvey 2000). In ventricular myocytes, it has been demonstrated that the stimulatory effect of M_2 receptor activation is due to opposing effects that G_i signaling has on the different isoforms of AC expressed in cardiac myocytes (Belevych et al. 2001). In addition to AC5 and AC6, there is also evidence for expression of AC4 and AC7 (Defer et al. 2000). While the activated α subunit of G_i inhibits AC5 and AC6, it has no effect on AC4 and AC7. On the other hand, AC4 and AC7 are stimulated by direct binding of $G\beta\gamma$ subunits (Sunahara and Taussig 2002). Therefore, it has been proposed that muscarinic stimulation can inhibit AC5 and AC6 while at the same time stimulating AC4 and AC7 (see Fig. 2). Furthermore, it has been proposed that muscarinic regulation cAMP inhibitory and stimulatory responses occur in distinct subcellular locations, and that the time-dependent flux of cAMP between these locations can explain the

complex temporal nature of the response (Iancu et al. 2007). In ventricular myocytes, the rebound stimulatory response has been shown to affect L-type Ca^{2+} channels as well as CFTR Cl^- channels. It has also been shown to stimulate spontaneous electrical activity and trigger delayed after depolarizations (Ehara and Mitsuiye 1984; Song et al. 1998). This suggests that the muscarinic receptor activation may contribute to arrhythmogenic activity associated with the complex interaction between parasympathetic and sympathetic stimulation of ventricular myocardium.

3.3 Other Muscarinic Responses in the Heart

In addition to the responses described above, high concentrations of muscarinic receptor agonists have also been reported to produce a positive inotropic effect associated with changes in intracellular Ca^{2+} secondary to an increase in intracellular Na^+ concentration (Korth and Kuhlkamp 1985; Korth et al. 1988). The increase in intracellular Na^+ has been attributed to activation of a tetrodotoxin (TTX)-insensitive Na^+ channel (Matsumoto and Pappano 1989). The resulting change in Na^+ gradient is believed to reduce the driving force for extrusion of intracellular Ca^{2+} by the Na/Ca exchanger (Saeki et al. 1997). This can then explain the increase in intracellular Ca^{2+} concentration and resulting change in force of contraction.

Most muscarinic responses in the heart have been attributed to activation of M_2 receptors. That includes activation of the TTX-insensitive Na^+ current by high agonist concentrations (Matsumoto and Pappano 1991). However, there is evidence for functional responses that are mediated by other types of muscarinic receptors. For example, even though the Na^+ current activated by high muscarinic agonist concentrations has been attributed to activation of M_2 receptors, the corresponding increase in intracellular Ca^{2+} and contractility are supposedly due to activation of M_1 receptors (Sharma et al. 1996). While the explanation for this apparent discrepancy is not clear, M_1 receptor activation has also been reported in to enhance L-type Ca^{2+} channel activity through a PLC-dependent mechanism (Gallo et al. 1993). On the other hand, M_3 receptor activation has been reported to activate a novel delayed rectifier-type K^+ current through a PLC-independent mechanism (Wang et al. 2004).

4 Vascular Muscarinic Responses

Muscarinic agonists can cause both contraction and relaxation of vascular tissue. The actual response can vary depending on the species and the anatomical location of the blood vessel involved, as well as whether or not the endothelial lining of the blood vessel is intact (Eglen et al. 1996). Relaxation is the primary response of most

blood vessels with an intact endothelium. Furchgott and Zawadzki were the first to demonstrate the role of the vascular endothelium in producing vasodilation of blood vessels in response to muscarinic agonist stimulation (Furchgott and Zawadzki 1980). This effect is typically mediated by an indirect mechanism that involves the release of an endothelium-derived relaxing factor (EDRF) following activation of M_3 receptors (Eglen et al. 1996; Eglen and Whiting 1990). Vascular endothelial cells can produce vasodilation by releasing multiple relaxing factors that act on vascular smooth muscle cells in a paracrine fashion. These include prostacyclin and endothelium-dependent hyperpolarization factor (EDHF). However, the most important factor involved in mediating the response to muscarinic agonists is NO (Furchgott and Vanhoutte 1989). This potent vasodilator is generated by eNOS, the isoform of nitric oxide synthase expressed constitutively in endothelial cells. The essential role of eNOS in muscarinic induced vasodilation is consistent with the significant reduction in the relaxation response to ACh observed in blood vessels obtained from eNOS knockout mice (Faraci and Sigmund 1999; Huang et al. 1995).

The signaling mechanism responsible for muscarinic receptor-dependent NO production in endothelial cells involves Ca^{2+} and calmodulin-dependent activation of eNOS (Dinerman et al. 1993). Consistent with this, the muscarinic receptors involved in vasorelaxation can trigger the release of Ca^{2+} from intracellular stores by stimulating PLC-dependent production of IP_3 (Adams et al. 1989). Once produced, NO can readily diffuse from the endothelial cells into adjacent smooth muscle cells. NO may then cause relaxation by one or more different actions. Perhaps the most important mechanism involves stimulation of soluble guanylyl cyclase activity (Pfeifer et al. 1998). This results in the production of cGMP, which can then activate PKG (see Fig. 3). Several mechanisms have been proposed to explain the ability of PKG to cause vascular smooth muscle relaxation (Faraci and Sigmund 1999; Hofmann et al. 2006).

Another indirect mechanism that may contribute to muscarinic relaxation of some blood vessels involves the inhibition of sympathetic neurotransmitter release (Vanhoutte and Shepherd 1983). Sympathetic stimulation of blood vessels causes potent vasoconstriction via the release of the neurotransmitter norepinephrine and subsequent activation of smooth muscle α-adrenergic receptors. Presynaptic inhibition of sympathetic neurotransmitter release by muscarinic agonists involves M_2 receptors (Eglen and Whiting 1990).

In addition to demonstrating the essential role that the vascular endothelium plays in agonist-induced relaxation of blood vessels, Furchgott and Zawadzki also demonstrated that in the absence of endothelium, muscarinic receptor activation can actually cause vascular smooth muscle contraction (Furchgott and Zawadzki 1980). This reflects the fact that vascular smooth muscle cells express G_q-coupled M_1 and M_3 muscarinic receptors capable of stimulating pharmacomechanical coupling. This involves the same PLC- and IP_3-dependent signaling mechanism that muscarinic receptors activate in endothelial cells. However, rather than stimulating NO production, the resulting rise in intracellular Ca^{2+} triggers myocyte contraction by regulating calmodulin-dependent activation of the myosin light chain kinase (see Fig. 3) (Horowitz et al. 1996).

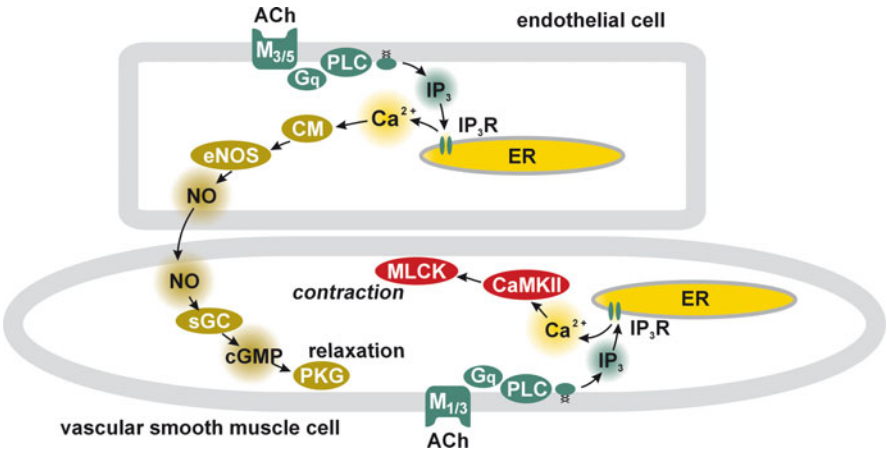

Fig. 3 Muscarinic signaling pathways in the vasculature. In endothelial cells, acetylcholine (ACh) acting through M_3 or M_5 receptors stimulates phospholipase C (PLC) activity through the G protein G_q. Subsequent production of inositoltriphosphate (IP_3) acts on the IP_3 receptor (IP_3R) in the endoplasmic reticulum to release Ca^{2+}. The resulting rise in cytosolic Ca^{2+} activates endothelial nitric oxide synthase (eNOS) via a calmodulin (CM)-dependent mechanism. Activation of eNOS leads to the production of nitric oxide (NO), which can diffuse into adjacent vascular smooth muscle cells, where it stimulates soluble guanylyl cyclase (sGC) to produce cGMP. Protein kinase G (PKG) activated by cGMP promotes relaxation. In vascular smooth muscle cells, ACh acting through M_1 or M_3 receptors stimulates PLC-dependent production of IP_3 and the subsequent release of Ca^{2+} from the ER. This results in Ca^{2+} and CM-dependent kinase (CamKII) activation of myosin light chain kinase (MLCK), which promotes contraction

Despite the significant effect that activation of muscarinic receptors can have on vascular function, parasympathetic stimulation does not play a significant role in autonomic regulation of blood flow in most vascular beds (Furchgott and Vanhoutte 1989). One notable exception to this generalization is in the cerebral circulation, where neurally released ACh is important in regulating vascular tone by causing endothelium-dependent vasodilation. In this case, however, the response to ACh involves the activation of M_5 receptors (Yamada et al. 2001).

5 Summary

Muscarinic receptor activation can regulate many different aspects of cardiovascular function. This review has focused primarily on normal physiological responses. However, there is a growing body of literature demonstrating that there are changes in muscarinic signaling that occur with age and various disease states (Dhein et al. 2001). There is also evidence that parasympathetic stimulation and muscarinic agonists can protect the heart from ischemic damage and prevent some of the deleterious effects associated with heart failure (Kakinuma et al. 2005; Katare

et al. 2009; Li et al. 2004). Because of this, a better understanding of muscarinic signaling pathways and the potential roles they play in regulating the heart and vasculature may provide new therapeutic strategies for treating cardiovascular disease.

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