British Journal of Pharmacology (2003) 139, 1074–1084
www.nature.com/bjp

REVIEW

Muscarinic regulation of cardiac ion channels

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The parasympathetic component of the autonomic nervous system plays an important role in the physiological regulation of cardiac function by exerting significant influence over the initiation as well as propagation of electrical impulses, in addition to being able to regulate contractile force. These effects are mediated in whole or in part through changes in ion channel activity that occur in response to activation of M_2 muscarinic cholinergic receptors following release of the neurotransmitter acetylcholine. The coupling of M_2 receptor activation to most changes in cardiac ion channel function can be explained by one of two general paradigms. The first involves direct G protein-dependent regulation of ion channel activity. The second involves indirect regulation of ion channel activity through modulation of cAMP-dependent responses. This review focuses on recent advances in our understanding of the mechanisms by which M_2 muscarinic receptor activation both inhibits and facilitates cAMP-dependent ion channel responses in the heart.
British Journal of Pharmacology (2003) **139,** 1074–1084. doi:10.1038/sj.bjp.0705338

Keywords: Muscarinic receptors; β-adrenergic receptors; adenylyl cyclase; cyclic AMP; nitric oxide; ion channels

Abbreviations: AC, adenylyl cyclase; ACh, acetylcholine; CM, calmodulin; EDS, early development stage; Iso, isoprenaline; LDS, late development stage; L-NAME, *N*-nitro-L-arginine-methyl-ester; L-NIO, L-*N*(5)-(1-iminoethyl)ornithine; L-NMMA, *N*-monomethyl-L-arginine; LY83583, 6-anilino-5,8-quinolinequinone; NO, nitric oxide; NOS1, type 1 nitric oxide synthase; NOS3, type 3 nitric oxide synthase; NOS3-KO, type 3 nitric oxide synthase knockout; ODQ, 1*H*-[1,2,4]oxadiazolo[4,3,-*a*] quinoxalin-1-one; PDE2, type 2 phosphodiesterase; PDE3, type 3 phosphodiesterase; PKA, protein kinase A; PTX, pertussis toxin

Introduction

The parasympathetic component of the autonomic nervous system plays an important role in the physiological regulation of cardiac function by exerting significant influence over the initiation as well as propagation of electrical impulses, in addition to being able to regulate contractile force (Löffelholz & Pappano, 1985; Hartzell, 1988). These effects are mediated in whole or in part through changes in ion channel activity that occur in response to activation of muscarinic cholinergic receptors following release of the neurotransmitter acetylcholine (ACh). The M_2 receptor is believed to be the predominant muscarinic receptor subtype expressed in cardiac muscle (Hulme *et al*., 1990; Dhein *et al*., 2001). Although there is evidence that other muscarinic receptor subtypes can produce effects in some cardiac preparations (Gallo *et al*., 1993; Colecraft *et al*., 1998; Shi *et al*., 1999a, b), their role in mediating physiological responses is not as well defined.

The coupling of M_2 receptor activation to most changes in cardiac ion channel function can be explained by one of two general paradigms. The first involves direct G protein-dependent regulation of ion channel activity. The second involves indirect regulation of ion channel activity through modulation of cAMP-dependent responses. The direct signaling pathway is involved in regulation of G protein coupled inward rectifying K^+ (GIRK) channels expressed primarily in atrial, sinoatrial node, and atrioventricular node cells. This signaling mechanism involves direct coupling of M_2 muscarinic receptors to GIRK channels *via* the pertussis toxin (PTX)-sensitive G protein G_i. Activation of this pathway is involved in parasympathetic inhibition or slowing of heart rate, and it may also contribute to slowing of impulse conduction through the atrioventricular node. Details concerning this signaling cascade can be found in recent reviews (Wickman & Clapham, 1995; Dascal, 1997; Mark & Herlitze, 2000). The remainder of the present article will focus on a description of the mechanisms by which M_2 muscarinic receptor activation both inhibits and facilitates cAMP-dependent ion channel responses in the heart.

Regulation of cardiac ion channels by cAMP

The sympathetic nervous system exerts significant influence over cardiac function, and it does so in large part by stimulating cAMP production. Therefore, the mechanisms by which muscarinic receptor activation regulates cAMP-dependent responses and the significance of the resulting effects can only be fully appreciated with an understanding how sympathetic stimulation affects the heart. Sympathetic innervation is found throughout the heart (Levy & Martin, 1989), and sympathetic stimulation can produce effects by activating both α- and β-adrenergic receptors on cardiac myocytes (Hartzell, 1988). However, it is the β-adrenergic signaling pathway that influences cardiac ion channel activity through the production of cAMP. Multiple subtypes of β-adrenergic

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Advance online publication: 23 June 2003

receptor are expressed in cardiac tissue. This includes β_1- and β_2-adrenergic receptors, which can both elicit responses *via* cAMP. However, the endogenous neurotransmitter norepinephrine selectively activates β_1-receptors, which are the predominant receptor subtype found in mammalian hearts (Kaumann, 1997; Brodde & Michel, 1999). Agonist binding leads to β-receptor activation of the stimulatory G protein, G_s, facilitating exchange of GTP for GDP and dissociation of the α subunit from the $\beta\gamma$ subunits (Fleming *et al*., 1992). The activated, GTP-bound $G_s\alpha$ subunit can directly interact with all isoforms of adenylyl cyclase (AC) expressed in cardiac tissue to stimulate the production of cAMP (Sunahara *et al*., 1996; Ishikawa & Homcy, 1997; Smit & Iyengar, 1998). This cyclic nucleotide can then alter ion channel function either directly by acting on the channel protein or indirectly by first activating protein kinase A (PKA). At least one cardiac ion channel appears to be affected by direct interaction of the channel protein with cAMP itself. That is the pacemaker channel expressed primarily in cells of the sinoatrial and atrioventricular node as well as specialized conducting cells of the adult mammalian heart (Accili *et al*., 2002). The more common scenario is for cardiac ion channels to be regulated by cAMP subsequent to PKA-dependent phosphorylation (Hartzell, 1988). Channels regulated in this manner include L-type Ca^{2+} channels (Tsien, 1973; Osterrieder *et al*., 1982; Bean *et al*., 1984; Kameyama *et al*., 1985; Trautwein *et al*., 1987), delayed rectifier-type K^+ channels (Tsien *et al*., 1972; Bennett & Begenisich, 1987; Walsh & Kass, 1988; Duchatelle-Gourdon *et al*., 1989; Giles *et al*., 1989; Harvey & Hume, 1989b; Marx *et al*., 2002; Kurokawa *et al*., 2003), cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channels (Bahinski *et al*., 1989; Harvey & Hume, 1989a; Harvey *et al*., 1990; Hwang *et al*., 1992), and voltage-dependent Na^+ channels (Hisatome *et al*., 1985; Cheng *et al*., 1991; Ono *et al*., 1993; Schreibmayer *et al*., 1994).

The effect of cAMP on pacemaker channels is believed to be a primary mechanism underlying sympathetic-induced increases in heart rate (Accili *et al*., 2002), and the effect of PKA activity on Ca^{2+} channels plays a key role in sympathetic regulation of cardiac muscle contraction (Tsien, 1977; Lindemann & Watanabe, 1989). Changes in Ca^{2+} as well as Na^+ channel activity may affect propagation of electrical impulses through changes in action potential upstroke velocity (Hisatome *et al*., 1985; Sperelakis & Josephson, 1989; Cheng *et al*., 1991). Ca^{2+} channel activity is also an important determinant of cardiac action potential duration, and in the absence of other changes sympathetic-induced enhancement of the L-type Ca^{2+} current can cause significant prolongation of the action potential. If left unchecked, this could potentially lead to the development of arrhythmias by triggering early after depolarizations and/or causing dispersion of repolarization and reentry. Although changes in Ca^{2+} influx may affect K^+ conductances independent of β-adrenergic stimulation (Kass & Tsien, 1976), the significance of cAMP-dependent regulation of K^+ and Cl^- channel activity is most likely to ensure that sympathetic stimulation does not produce untoward increases in action potential duration (Kass & Wiegers, 1982; Bennett & Begenisich, 1987; Harvey & Hume, 1989a; Harvey *et al*., 1990). The importance of balancing the effects of sympathetic stimulation on action potential duration is illustrated by the role that defects in cAMP-dependent regulation of delayed rectifier K^+ channel function play in triggering lethal arrhythmias associated with increases in sympathetic tone in certain forms of long QT syndrome (Marx *et al*., 2002; Kurokawa *et al*., 2003).

Through one or more indirect signaling pathways, M_2 muscarinic receptor stimulation can modulate the cAMP-dependent responses to β-adrenergic receptor activation described above. The ability of the muscarinic agonist ACh to produce both inhibitory and stimulatory responses is illustrated in Figure 1. A submaximally stimulating concentration of the β-adrenergic receptor agonist isoprenaline enhances L-type Ca^{2+} channel activity. Subsequent exposure to ACh results in inhibition of this cAMP-dependent response, and

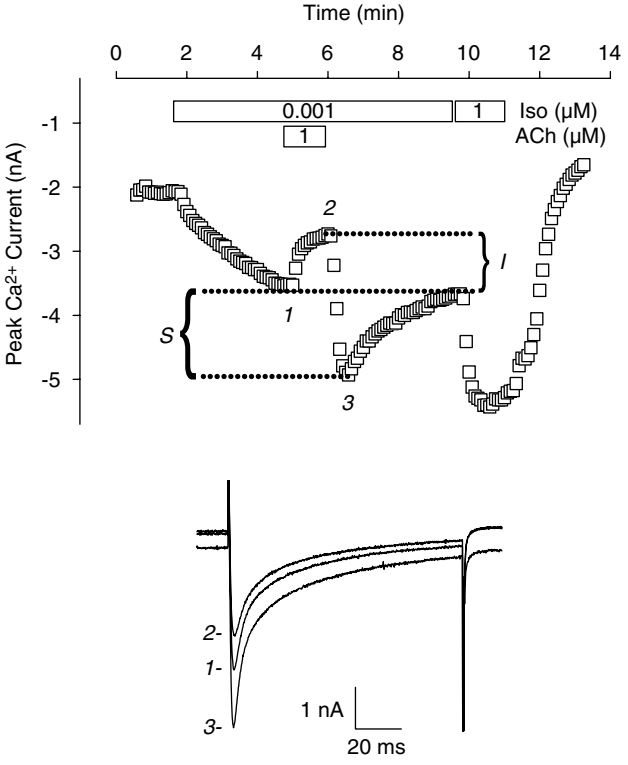

Figure 1 Muscarinic inhibition and stimulation of the L-type Ca^{2+} current. Top panel: Time course of changes in the magnitude of the L-type Ca^{2+} current recorded from an isolated guinea-pig ventricular myocyte following exposure to the muscarinic receptor agonist ACh in the presence of a submaximally stimulating concentration of the β-adrenergic receptor agonist isoprenaline (Iso). Currents were elicited by applying 100 ms depolarizing voltage clamp steps to a test potential of 0 mV following a 40 ms conditioning pulse to -30 mV from a holding potential of -80 mV. The Ca^{2+} current was measured as the absolute magnitude of the peak inward current elicited during the test pulse. Muscarinic inhibition of the β-adrenergic response (I) is observed in the presence of ACh. Washout of ACh in the continued presence of β-adrenergic stimulation reveals the rebound stimulatory response (S) to muscarinic receptor activation. Bottom panel: Current traces recorded at time points indicated in top panel. (1) Steady-state response to submaximally stimulating concentration of Iso, (2) steady-state inhibitory effect of ACh in the presence of Iso, and (3) peak rebound stimulatory effect following washout of ACh in the continued presence of Iso. Changes in the steady-state pre- and post-test pulse currents are due to parallel activation of the cAMP regulated, time-independent Cl^- current. The Cl^- current did not affect measurement of the Ca^{2+} current at the test potential since the Cl^- equilibrium potential was set at 0 mV. Further experimental details are as described in Belevych *et al*. (2001).

washout of the muscarinic agonist reveals a stimulatory rebound increase in Ca^{2+} current magnitude.

Muscarinic inhibition of cAMP-dependent responses

The indirect effect most often associated with muscarinic receptor stimulation is an inhibition of cAMP-dependent responses. The ability of M_2 muscarinic receptor activation to inhibit cAMP-dependent responses has been termed 'accentuated antagonism' (Levy, 1971). More specifically, this refers to the fact that the inhibitory response to muscarinic receptor activation is more prominent or becomes evident in the presence of sympathetic or β-adrenergic receptor stimulation. This is especially true concerning the effects of parasympathetic stimulation on ventricular function. In fact, there is the common misconception that muscarinic receptors are not expressed in ventricular tissue, since muscarinic agonists have little or no effect on ventricular function under basal conditions (Levy, 1995). Yet, this is clearly not the case. There is significant parasympathetic innervation of the ventricular myocardium (Standish *et al*., 1994), and muscarinic receptors are expressed throughout all areas of the heart, including the ventricles (Löffelholz & Pappano, 1985). However, in most mammals, muscarinic responses are only observed in adult ventricular myocytes under conditions where cAMP production has been enhanced above basal levels, through a mechanism such as β-adrenergic receptor activation (Hartzell, 1988; McDonald *et al*., 1994). 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 (Iijima *et al*., 1985; DiFrancesco & Tromba, 1988; Petit-Jacques *et al*., 1993; Wang & Lipsius, 1995; 1996; Wang *et al*., 1997; 1998; Vandecasteele *et al*., 1998). This is consistent with the idea that even under basal conditions atrial cells exhibit enhanced adenylyl cyclase activity resulting in increased cAMP production, which can then be inhibited by muscarinic receptor activation (Méry *et al*., 1997).

Muscarinic inhibition of cAMP production

Identifying the mechanism by which M_2 receptor activation can antagonize cAMP-dependent responses has been an active area of investigation for more than four decades. Early studies demonstrated that exposure to ACh can reduce cAMP levels in cardiac tissue (Murad *et al*., 1962; Löffelholz & Pappano, 1985; Hartzell, 1988). This effect was attributed to inhibition of AC activity by a mechanism involving a PTX-sensitive G protein, (Hazeki & Ui, 1981; 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; Smit & Iyengar, 1998). This supports the idea that ACh can antagonize β-adrenergic responses by inhibiting cAMP synthesis (Figure 2).

Other studies have suggested that the inhibitory effects of ACh do not always correlate with changes in cAMP levels (Watanabe & Besch, 1975; Keely Jr., *et al*., 1978; Biegon & Pappano, 1980; Lindemann & Watanabe, 1989; Schmied & Korth, 1990; Gupta *et al*., 1994; Zhang & MacLeod, 1996). This has led some to speculate that ACh might antagonize cAMP-dependent responses by stimulating phosphatase activity and enhancing protein dephosphorylation (Ahmad *et al*., 1989; Gupta *et al*., 1994; Herzig *et al*., 1995; Stemmer *et al*., 2000; Shen & Pappano, 2002). Consistent with this idea, there is evidence that M_2 receptor stimulation can produce disinhibition of protein phosphatase 1 (PP1) through phosphorylation of regulatory peptide inhibitor-1 (Ahmad *et al*., 1989). 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, it 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 & Tortora, 1991).

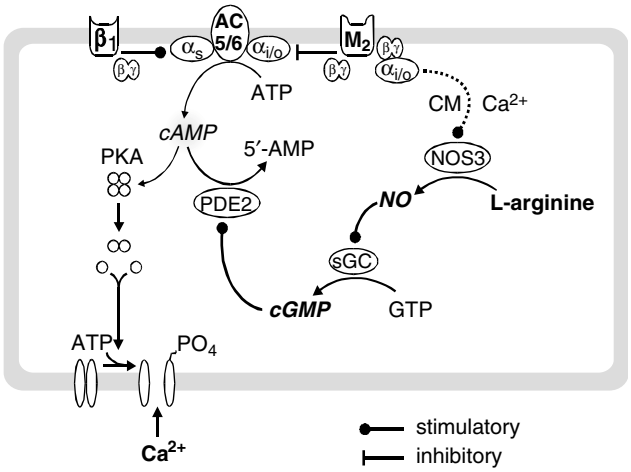

Figure 2 Proposed signaling pathways responsible for M_2 muscarinic receptor inhibition of cAMP-dependent ion channel responses. Muscarinic inhibitory responses may be mediated by directly inhibiting AC *via* the α subunit ($\alpha_{i/o}$) of the PTX-sensitive G proteins G_i or G_o or by stimulating PDE2 *via* production of NO and cGMP. In ventricular myocytes, muscarinic responses are only observed in the presence of agonists that stimulate cAMP production. β_1-adrenergic receptors stimulate cAMP synthesis by directly activating AC *via* the α subunit (α_s) of the stimulatory G protein G_s. See text for details; sGC, soluble guanylyl cyclase.

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 not be detected at the tissue or even cellular level. Support for this conclusion comes from work demonstrating that ACh affects ventricular Ca^{2+} channels only when the muscarinic agonist is applied to the same subcellular location as the β-adrenergic agonist being used to elicit the cAMP-dependent response (Jurevicius & Fischmeister, 1996). Localization of muscarinic responses to discrete subcellular domains may also help explain how M_2 receptor activation differentially regulates cAMP-dependent responses to β_1- and β_2-adrenergic receptor stimulation (Aprigliano *et al*., 1997). With the development of techniques that allow the subcellular imaging of cAMP activity (Goaillard *et al*., 2001; Zaccolo & Pozzan, 2002), it may soon be possible to identify the precise

subcellular pattern of changes in cAMP levels that occur following activation of different G protein coupled receptors.

Potential role for NO and cGMP

Early studies also demonstrated that exposure to ACh is associated with the production of cGMP in cardiac tissue (George *et al*., 1970; 1972; Watanabe & Besch, 1975). However, a clear role for cGMP in mediating muscarinic responses originally fell from favor, at least in part, because there was never a consistent correlation between ACh effects and cGMP levels (Löffelholz & Pappano, 1985; Hartzell, 1988). Nevertheless, it is clear that in at least some cardiac myocyte preparations, exogenous cGMP or cGMP analogs can antagonize cAMP-mediated ion channel responses. Such effects have been attributed to exogenous cGMP stimulating the activity of type 2 phosphodiesterase (PDE2), resulting in an increase in cAMP breakdown (Hartzell & Fischmeister, 1986; 1987; Fischmeister & Hartzell, 1987; Simmons & Hartzell, 1988; Méry *et al*., 1995; Wang & Lipsius, 1995; Han *et al*., 1996; Vandecasteele *et al*., 2001). In other preparations, the inhibitory effects of exogenous cGMP have been explained by activation of protein kinase G (Wahler & Sperelakis, 1985; Thakkar *et al*., 1988; Levi *et al*., 1989; Lohmann *et al*., 1991; Ono & Trautwein, 1991; Tohse & Sperelakis, 1991; Mubagwa *et al*., 1993; Levi *et al*., 1994; Shirayama & Pappano, 1996; Imai *et al*., 2001; Shen & Pappano, 2002). Despite these findings, evidence linking either of these potential effects of cGMP to muscarinic receptor activation has been inconsistent.

Renewed interest in cGMP and its possible role in mediating muscarinic responses followed the discovery of nitric oxide (NO) as the regulator of soluble guanylyl cyclase activity in a variety of mammalian cell types (Moncada *et al*., 1991; Snyder & Bredt, 1991). Subsequently, it was demonstrated that muscarinic agonists can stimulate the production of NO by cardiac myocytes, and exposure to compounds that inhibit NO synthase (NOS), the enzyme that converts L-arginine to NO, can block both the production of NO and functional responses to muscarinic receptor activation (Balligand *et al*., 1993). It was later proposed that NO plays an obligatory role in mediating muscarinic inhibition of cAMP-dependent cardiac ion channel responses through a mechanism that involves cGMP-dependent regulation of PDE2 activity (Han *et al*., 1994; Balligand *et al*., 1995; Han *et al*., 1995; 1996; 1998a, b) (Figure 2). The ability of higher concentrations of NO donors to mimic muscarinic inhibition of cAMP-dependent ion channel responses is consistent with this hypothesis. However, it does not prove that the muscarinic signaling pathway actually involves the production of NO. More direct evidence comes from studies demonstrating that pharmacologic inhibitors of NOS, guanylyl cyclase, and PDE2 are able to block muscarinic antagonism of cAMP-dependent ion channel responses (Mubagwa *et al*., 1993; Han *et al*., 1994; 1995; 1996; Levi *et al*., 1994; Balligand *et al*., 1995; Gallo *et al*., 1998; Imai *et al*., 2001). Most studies supporting the idea that NO and cGMP play an obligatory role in muscarinic inhibition of cAMP-dependent ion channel activity have been conducted using myocytes from sinoatrial and atrioventricular node preparations (Han *et al*., 1994; 1995; 1996; 1997; 1998a, b). However, it has also been suggested that the same signaling mechanism is responsible for analogous muscarinic effects in ventricular myocytes (Mubagwa *et al*., 1993; Levi *et al*., 1994; Balligand *et al*., 1995; Wahler & Dollinger, 1995; Kelly *et al*., 1996; Gallo *et al*., 1998).

Evidence against NO and cGMP

Despite the evidence supporting a role for NO in mediating muscarinic inhibitory responses in ventricular myocytes, several studies using a similar pharmacological approach have reached the opposite conclusion. As in the work described above, these studies have relied on the use of exogenous NO donors to mimic or inhibitors of NOS and/or guanylyl cyclase activity to block muscarinic responses. However, unlike the work described above, many of these compounds were found to have either no effect or secondary effects that confounded interpretation of the results.

The ability of NO donors to mimic muscarinic inhibition of β-adrenergic responses has been used as indirect evidence that NO mediates these muscarinic effects. However, NO itself is an oxidizing agent and some NO donors, in addition to producing NO, also release other reactive species (Stamler, 1994). In at least one study, NO donors were found to antagonize β-adrenergic regulation of cardiac ion channel responses not by mimicking muscarinic responses, but by oxidizing the β-adrenergic agonist (Zakharov *et al*., 1996). In other studies, NO donors have been found to alter the activity of ion channels through direct oxidation or nitrosylation of the channel protein (Campbell *et al*., 1996; Hu *et al*., 1997). Therefore, conclusions concerning the mechanisms responsible for changes in channel function caused by exposure to exogenous NO can be complicated by actions unrelated to activation of guanylyl cyclase.

The ability of NOS inhibitors to antagonize muscarinic inhibition of β-adrenergic responses has been used as evidence to support the conclusion that NO production is obligatory in mediating muscarinic responses. Compounds most commonly used for this purpose are derivatives of L-arginine, which act as competitive substrate inhibitors of NOS. However, at least one such compound, *N*-nitro-L-arginine-methyl-ester (L-NAME), has also been found to act as a muscarinic receptor antagonist (Buxton *et al*., 1993). Although there is no evidence to suggest that the use of L-NAME has led to any erroneous conclusions concerning the role of NO in muscarinic regulation of cardiac ion channels, this highlights another example of the potential complications associated with the nonspecific effects of a pharmacological tool that should be avoided.

Another factor to consider when investigating the role of NO in mediating muscarinic responses is that elimination of NOS activity has been reported to increase the sensitivity of some cardiac preparations to β-adrenergic stimulation (Balligand *et al*., 1993; Balligand, 1999). This is significant not only because it demonstrates that NO production, independent of muscarinic receptor stimulation, can play a role in regulating β-adrenergic responses, but also because muscarinic antagonism of β-adrenergic responses is functionally competitive. Increasing the level of β-adrenergic stimulation can overcome the inhibitory effect that a given concentration of a muscarinic agonist has on cardiac ion channel function (Hescheler *et al*., 1986; Zakharov *et al*., 1995). Owing to this fact, anything that increases the sensitivity of cardiac myocytes to β-adrenergic stimulation should reduce their apparent sensitivity to muscarinic inhibition, independent of any direct effect it might

have on the muscarinic signaling pathway. Therefore, elimination of basal NO production has the potential of attenuating responses to muscarinic receptor activation at least in part by increasing β-adrenergic responsiveness. However, the fact that several studies have demonstrated that NOS inhibitors have no effect at all on muscarinic inhibition of cAMP-regulated ion channel activity suggests not only that these muscarinic responses do not involve NO production, but also that basal NO production does not affect the β-adrenergic sensitivity of all cardiac myocyte preparations (Méry *et al*., 1996; Zakharov *et al*., 1996; Vandecasteele *et al*., 1998; Belevych *et al*., 2001; Bett *et al*., 2002).

The ability of guanylyl cyclase inhibitors to block muscarinic inhibition of β-adrenergic responses has also been used as evidence that cGMP production is directly involved in mediating muscarinic responses. However, some of the commonly used pharmacologic agents have confounding side effects. For example, 6-anilino-5,8-quinolinequinone (LY83583) can inhibit soluble guanylyl cyclase activity, and it has been reported to block muscarinic- and NO-dependent inhibition of β-adrenergically regulated ion channel activity in adult ventricular myocytes (Méry *et al*., 1993; Han *et al*., 1995; Wahler & Dollinger, 1995; Imai *et al*., 2001). However, LY83583 can also significantly potentiate responses to β-adrenergic agonists in the absence of ACh (Zakharov *et al*., 1996; Abi-Gerges *et al*., 1997b; Imai *et al*., 2001). This is another example of where understanding the competitive nature of the interaction between muscarinic and β-adrenergic responses becomes important when interpreting results. The ability of LY83583 to antagonize muscarinic inhibitory effects might then be explained by the ability of this compound to increase the sensitivity of the cell to β-adrenergic stimulation, rather than its ability to directly block the muscarinic signaling pathway (Zakharov *et al*., 1996). It is interesting to note, however, that this nonspecific effect of LY83583 only appears to be observed when the drug is applied extracellularly (Imai *et al*., 2001).

Methylene blue is another commonly used guanylyl cyclase inhibitor, and it too has been shown to block ACh inhibition of β-adrenergically regulated cardiac ion channel activity (Levi *et al*., 1994; Han *et al*., 1995; Vandecasteele *et al*., 1998). However, methylene blue can also act as an antagonist at M_2 receptors, complicating the interpretation of any results involving the use of muscarinic receptor agonists (Abi-Gerges *et al*., 1997a; Pfaffendorf *et al*., 1997). Nevertheless, it appears that methylene blue can still be used to verify the role of guanylyl cyclase in mediating muscarinic responses, but only if it can be applied selectively to the intracellular compartment (Han *et al*., 1995).

Studies employing pharmacological tools have provided important insight into the mechanisms by which NO and cGMP regulate cAMP-dependent ion channel responses, but because of potential complications like those described above, they may not provide definitive evidence for or against a role for these second messengers in mediating muscarinic responses. To address this issue, several groups have employed the use of mice in which there has been targeted disruption of the gene responsible for expression of endothelial or type 3 NOS (NOS3), the predominant isoform of NOS constitutively expressed in cardiac myocytes (Han *et al*., 1998b; Vandecasteele *et al*., 1999; Belevych & Harvey, 2000; Gödecke *et al*., 2001). Although there is also evidence for expression of neuronal or type 1 NOS (NOS1) in the heart of some species, its role appears to be limited to the regulation of ryanodine receptor function and Ca^{2+} release from the sarcoplasmic reticulum (Barouch *et al*., 2002). NOS3 is believed to be the isoform important in regulating cAMP-dependent responses in the heart (Balligand *et al*., 1995). Consistent with this hypothesis, the ability of muscarinic agonists to stimulate guanylyl cyclase activity is absent in ventricular myocytes isolated from NOS3 knockout (NOS3-KO) animals. It was also reported in the same study that the ability of muscarinic agonists to inhibit β-adrenergic regulation of L-type Ca^{2+} channel activity is absent in these cells (Han *et al*., 1998b). However, at least three other groups have found that muscarinic responses are unaltered in ventricular myocytes isolated from NOS3-KO animals (Vandecasteele *et al*., 1999; Belevych & Harvey, 2000; Gödecke *et al*., 2001).

A difference in the age of the animals used has been offered as one explanation for the discrepant results (Balligand, 1999; Hare & Stamler, 1999). The absence of muscarinic regulation of ion channel responses was reported in myocytes isolated from neonates to animals 2 months of age (Han *et al*., 1998b). Intact muscarinic ion channel responses were reported in myocytes isolated from animals that were either 2–4 months of age (Belevych *et al*., 2001) or 3–6 months of age (Vandecasteele *et al*., 1999; Gödecke *et al*., 2001). Older NOS3-KO animals develop cardiac hypertrophy secondary to hypertension (Yang *et al*., 1999), and it has been suggested that hypertrophy upregulates GIRK channel expression, which somehow complicates interpretation of the effect that muscarinic receptor stimulation has on Ca^{2+} channel regulation (Balligand, 1999). Although other factors associated with the development of hypertrophy have been reported to alter the expression of certain types of voltage-dependent K^+ channels, there is no direct evidence that hypertrophy upregulates GIRK channel expression in ventricular myocytes of NOS-KO animals. Even if hypertrophy were to have such an effect, it could not explain the fact that muscarinic receptor activation still inhibited β-adrenergic regulation of the L-type Ca^{2+} current recorded under conditions where all K^+ conductances were blocked (Vandecasteele *et al*., 1999; Belevych & Harvey, 2000; Gödecke *et al*., 2001). Nevertheless, the fact that the hearts of NOS-KO animals do undergo hypertrophic changes illustrates the fact that the use of these genetically engineered animals is not without potential complications.

Developmental regulation of muscarinic signaling pathways

It is conceivable, however, that developmental changes in the signaling pathways linking M_2 muscarinic receptors to regulation of cAMP-dependent ion channel responses might explain at least some of the differences in muscarinic responses observed when using NOS3-KO animals of different ages. Consistent with the premise that NO might play a more important role in less mature myocytes, Ji *et al*. (1999) reported that in embryonic stem cells differentiated into cardiac myocytes, muscarinic inhibition of cAMP-dependent responses involved the NO/cGMP signaling pathway in cells from an early development stage (EDS), but not in cells from a late development stage (LDS). Based on these results, the authors concluded that the NO/cGMP signaling pathway is initially involved in muscarinic inhibition of cAMP-dependent

ion channel responses, but that there is a switch to direct G_i-mediated inhibition of AC during maturation. While it is still unclear why there might be a need for unique muscarinic signaling mechanisms during different stages of development, this is an intriguing hypothesis. The ability of exogenous NO to inhibit basal Ca^{2+} channel activity through a guanylyl cyclase-dependent mechanism in neonatal ventricular myocytes, but not in adult ventricular myocytes, is consistent with this theory (Vulcu *et al*., 2000). The ability of NO to inhibit basal channel activity in EDS stem cells and neonatal ventricular myocytes can be explained by the observation that they actually exhibit a phenotype similar to atrial and sinoatrial node cells, where basal Ca^{2+} channel activity can be inhibited by muscarinic receptor stimulation, whereas LDS cells exhibit a phenotype more like that found in adult ventricular myocytes, where basal Ca^{2+} channel activity is typically insensitive to inhibition by muscarinic agonists. These parallels raise the possibility that that there may even be differences in muscarinic signaling mechanisms between atrial and ventricular myocytes.

Muscarinic facilitation of cAMP-dependent responses

Despite the fact that muscarinic receptor activation can potently antagonize β-adrenergic responses, it is also known, but perhaps not as widely recognized, that parasympathetic input to the heart produces significant stimulatory effects. One clear manifestation of such an effect is the rebound increase in heart rate as well as contractility that can be observed immediately following termination of vagal stimulation or cessation of exposure to ACh (Hollenberg *et al*., 1965; Levy, 1971; Burke & Calaresu, 1972; Loeb & Vassalle, 1978; Gilmour & Zipes, 1985; Endoh, 1999). One suggestion has been that these stimulatory effects of ACh might be due to the release of catecholamines from either presynaptic sympathetic fibers or extraneuronal stores (Loeb & Vassalle, 1979). However, rebound responses to ACh can be observed in isolated cardiac myocytes (Tareen *et al*., 1991; Ono & Noma, 1994; Wang & Lipsius, 1995; Wang & Lipsius, 1996; Wang *et al*., 1997; 1998; Zakharov & Harvey, 1997; Song *et al*., 1998; Belevych & Harvey, 2000; Belevych *et al*., 2001).

In atrial myocytes, exposure to ACh produces a slight inhibition of the basal L-type Ca^{2+} current, and subsequent washout of ACh causes a marked stimulation of the current beyond the magnitude observed prior to exposure to ACh (Wang & Lipsius, 1995; Wang *et al*., 1997; 1998). Although this rebound response in atrial cells can be observed in the absence of β-adrenergic stimulation, evidence suggests that it is due to facilitation of a cAMP-dependent mechanism made possible because of the high basal level of cAMP production in these cells. The conclusion that atrial rebound responses involve the facilitation of a cAMP-dependent mechanism is supported by the fact that ACh-induced rebound stimulation of Ca^{2+} channel activity is blocked by inhibitors of PKA (Wang & Lipsius, 1995; Wang *et al*., 1998). Furthermore, ACh withdrawal causes rebound stimulation of other cAMP-regulated ion channels, including those responsible for the pacemaker current (Wang & Lipsius, 1996). In fact, rebound stimulation of the pacemaker current in sinoatrial node cells has been proposed to explain the phenomenon of postvagal tachycardia, which is the rebound increase in heart rate observed upon termination of vagal stimulation (Wang & Lipsius, 1996). Although ACh-induced hyperpolarization of the resting membrane potential caused by activation of GIRK channels can lead to enhanced activation of the pacemaker current *via* a cAMP-independent mechanism, theoretical calculations suggest that this is likely to play a relatively minor role in decreasing cycle length in sinoatrial node cells following termination of vagal stimulation (Dokos *et al*., 1996).

Muscarinic receptor activation elicits the same type of stimulatory response in ventricular cells (see Figure 1). ACh-induced rebound stimulation of cAMP-regulated Cl^- and Ca^{2+} channel activity has been described in isolated ventricular myocytes, but because basal levels of cAMP production are low in these cells, ACh-induced stimulatory effects are not observed in the absence of an agonist that stimulates cAMP production *via* a G_s-dependent mechanism. In fact, the magnitude of the ACh-induced stimulatory response is enhanced by increasing the level of G_s-dependent stimulation. However, the ACh-induced stimulatory response and maximal G_s-dependent stimulatory responses are not additive. All of this supports the idea that the stimulatory effect of ACh is due to facilitation of cAMP-dependent responses (Zakharov & Harvey, 1997; Belevych & Harvey, 2000; Belevych *et al*., 2001).

ACh-induced facilitation of cAMP-dependent responses is mediated by M_2 receptors, the same muscarinic receptor subtype that is involved in inhibition of cAMP-dependent responses (Wang & Lipsius, 1995; Zakharov & Harvey, 1997). Furthermore, both the stimulatory and inhibitory effects are mediated *via* PTX-sensitive G proteins (Wang & Lipsius, 1995; Zakharov & Harvey, 1997; Belevych *et al*., 2001). But even though both responses are initiated by a common mechanism, they exhibit distinct kinetic differences. The inhibitory effect turns on and off rapidly, while the stimulatory effect turns on and off at least an order of magnitude more slowly (Zakharov & Harvey, 1997). Furthermore, the net response observed during exposure to ACh is typically inhibitory. However, upon termination of muscarinic receptor activation, the inhibitory effect reverses more rapidly revealing the stimulatory effect.

In addition to being able to explain physiologic phenomena such as postvagal tachycardia (Wang & Lipsius, 1996), the stimulatory effects associated with rebound stimulation are also believed to play a role in triggering certain types of arrhythmogenic mechanisms (Wang *et al*., 1997; Song *et al*., 1998). However, it should not be assumed that ACh-induced stimulatory effects are only observed upon termination of muscarinic receptor activation. Just because the net response observed in the presence of ACh is inhibitory does not mean that the stimulatory effect is not important under those conditions as well. The net effect is most likely a balance between the stimulatory and inhibitory actions of ACh. In fact, continued exposure to ACh results in a time-dependent relaxation of its inhibitory actions (Zakharov & Harvey, 1997; Endoh, 1999). While this phenomenon might be attributable to muscarinic receptor desensitization, a more slowly developing stimulatory effect could also play a role.

Role of NO and cGMP

Ono & Noma (1994) originally suggested that ACh-induced rebound responses might be explained by the production of

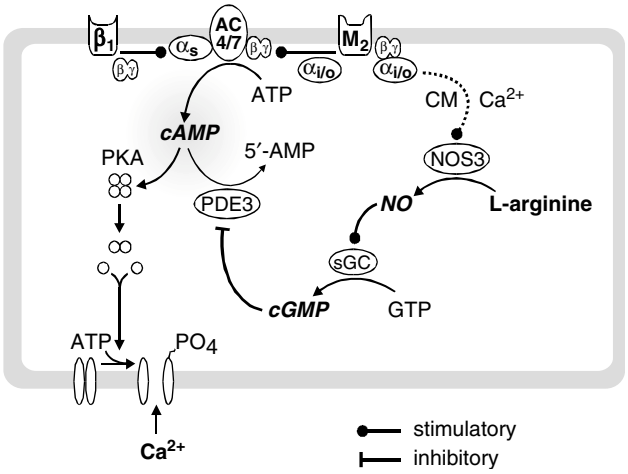

Figure 3 Proposed pathways responsible for M_2 muscarinic receptor stimulation of cAMP-dependent ion channel responses. Muscarinic stimulatory responses may be mediated by direct activation of AC *via* the $\beta\gamma$ subunits of a PTX-sensitive G protein G_i or G_o or by inhibition of PDE3 *via* production of nitric oxide (NO) and cGMP. Direct stimulation of AC by $\beta\gamma$ subunits is only observed under conditions, such as the presence of a β_1-adrenergic receptor agonist, that can activate AC *via* direct interaction of the α subunit (α_s) of the stimulatory G protein G_s. See text for details; sGC, soluble guanylyl cyclase.

cGMP. This hypothesis was supported by the observation that low concentrations of NO donors and exogenous cGMP can actually facilitate cAMP-dependent stimulation of cardiac ion channels (Ono & Trautwein, 1991; Ono *et al*., 1992; Méry *et al*., 1993; Kirstein *et al*., 1995). Subsequently, Lipsius and co-workers (Wang *et al*., 1998) demonstrated that in cat atrial myocytes, ACh-induced rebound responses could be blocked by (1) W-7, a calmodulin (CM) inhibitor, (2) L-*N*(5)-(1-iminoethyl)ornithine (L-NIO), an inhibitor of constitutive NOS activity, (3) 1*H*-[1,2,4]oxadiazolo[4,3,-*a*]quinoxalin-1-one (ODQ), a soluble guanylyl cyclase inhibitor, and (4) milrinone, an inhibitor of type 3 PDE (PDE3) activity. These results support the conclusion that ACh-induced rebound stimulation of atrial responses is mediated through the Ca^{2+}-CM-dependent activation of NOS, NO-dependent stimulation of soluble guanylyl cyclase, and cGMP-dependent inhibition of PDE3 (Figure 3). Both PDE2 and PDE3 are expressed in cardiac myocytes (Fischmeister & Hartzell, 1991). However, cGMP elicits opposing regulatory effects on these two PDE isoforms. Thus, it is conceivable for muscarinic receptor activation to produce both facilitation as well as inhibition of cAMP-dependent ion channel responses *via* a NO/cGMP-dependent mechanism. Whether one would expect to see an inhibitory and/or stimulatory response would then depend on the relative level of expression of the different PDE isoforms, and whether or not they are coupled to muscarinic receptor activation.

Role of direct regulation of AC activity

Despite evidence that the NO/cGMP signaling pathway is involved in mediating muscarinic stimulatory responses in atrial myocytes, other studies have demonstrated that the NO/cGMP signaling pathway is not important in mediating stimulatory responses in ventricular cells. This includes the inability to modify rebound responses in guinea-pig ventricular myocytes by (1) buffering intracellular Ca^{2+} to prevent Ca^{2+}-CM-dependent activation of NOS, (2) inhibiting NOS activity with the L-arginine analog *N*-monomethyl-L-arginine (L-NMMA), (3) inhibiting guanylyl cyclase activity with ODQ, and (4) inhibiting PDE3 activity with milrinone (Zakharov & Harvey, 1997; Belevych *et al*., 2001). Furthermore, it has been demonstrated that muscarinic stimulatory responses are intact in ventricular myocytes isolated from NOS3-KO mice (Belevych & Harvey, 2000).

Alternatively, it has been demonstrated that the stimulatory effect of M_2 receptor activation in ventricular myocytes is due to opposing effects of PTX-sensitive G proteins on the different isoforms of AC expressed in cardiac myocytes (Belevych *et al*., 2001) (Figure 3). In addition to AC5 and AC6, there is also evidence for expression of AC4 and AC7 in the heart (Defer *et al*., 2000). Previously, most investigators had assumed that the only isoforms of any consequence are AC5 and AC6. This is based on the observation that the messenger RNA levels for these isoforms of AC appear to be the most abundant (Ishikawa & Homcy, 1997). Furthermore, it is known that muscarinic receptor activation can directly inhibit cardiac AC activity *via* the PTX-sensitive G protein G_i (Hartzell, 1988), and G_i inhibits AC5 and AC6, but not AC4 and AC7 (Sunahara *et al*., 1996; Smit & Iyengar, 1998). Inhibition of AC5 and AC6 involves direct binding of the activated $G_i\alpha$ subunit. AC4 and AC7 are structurally similar to AC2, and activated $G_i\alpha$ does not inhibit AC2. In fact, unlike AC5 and AC6, AC2 and AC4 can actually be stimulated by direct binding of $G\beta\gamma$ (Gao & Gilman, 1991; Tang & Gilman, 1991; Federman *et al*., 1992; Chen *et al*., 1995). However, this stimulatory effect of $G\beta\gamma$ involves a conditional type of regulation in that it only occurs in the presence of activated $G_s\alpha$. Therefore, one might predict that in the presence of activated $G_s\alpha$, muscarinic stimulation would inhibit AC5 and AC6 but stimulate AC4 and AC7 (Figure 4). Furthermore, the faster onset and offset of the inhibitory effects are consistent

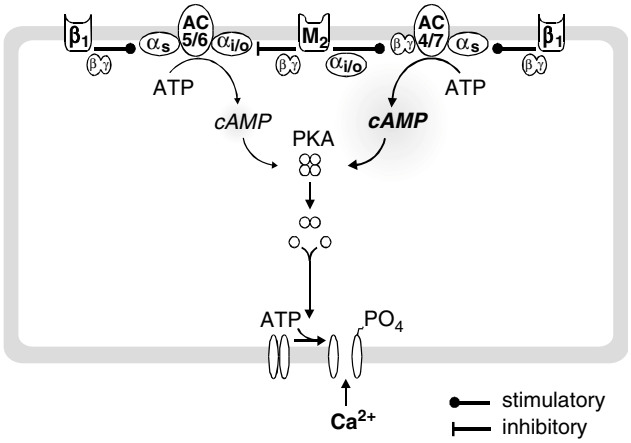

Figure 4 Proposed mechanism by which M_2 muscarinic receptor activation can produce both inhibitory and stimulatory responses through the differential regulation of specific AC isoforms. Muscarinic inhibitory effects can be produced by inhibition of AC types 5 and/or 6 (AC5/6) *via* direct interaction with the α subunit ($\alpha_{i/o}$) of a PTX-sensitive G protein G_i or Go. Muscarinic stimulatory effects can be produced by activation of AC types 4 and/or 7 (AC4/7) *via* direct interaction with the $\beta\gamma$ subunits of G_i or Go. See text for details.

with the observation that the Gα binding regions of AC can act as regulators of G protein signaling. They possess guanine nucleotide exchange factor properties as well as GTPase activating properties (Scholich *et al*., 1999; Wittpoth *et al*., 2000). This would be expected to speed the onset and offset of muscarinic inhibitory effects mediated by $G_i\alpha$, but not the stimulatory effects mediated by $G\beta\gamma$.

Consistent with a model that involves conditional regulation of AC4 and/or AC7, it has been demonstrated that in ventricular myocytes, ACh-induced rebound stimulation of ion channel activity is only observed under conditions expected to generate activated $G_s\alpha$ (Belevych *et al*., 2001). This includes β-adrenergic or H_2 histamine receptor activation or pretreatment with cholera toxin. Direct stimulation of AC in a $G_s\alpha$-independent manner using forskolin does not enable ACh to produce its stimulatory effect. Furthermore, the stimulatory response to ACh can be blocked by dialysis of ventricular myocytes with QEHA, a 27 amino-acid peptide representing the $\beta\gamma$ binding region of AC2 (Chen *et al*., 1995). In addition, it was found that when the stimulatory component of the ACh response is blocked, the ability of ACh to inhibit cAMP-dependent ion channel responses is enhanced (Belevych *et al*., 2001). This supports the idea that the net response observed in the presence of ACh is a balance between the inhibitory and stimulatory actions elicited by M_2 receptor activation (see Figure 4).

Summary

It is quite clear that modulation of cAMP-dependent ion channel responses by M_2 muscarinic receptors plays an important role in regulating cardiac function. Although the antagonistic interaction between sympathetic and parasympathetic regulation of such responses has long been considered a classic physiological example of yin-yang, it has become apparent that the net response to muscarinic receptor activation actually represents a balance between inhibition as well as facilitation of cAMP-dependent responses. However, the mechanisms by which these effects are mediated still require further investigation. There is undisputable evidence that NO and cGMP can significantly influence cAMP-dependent responses in the heart, but there is still some question as to whether or not they are involved in mediating either the stimulatory or the inhibitory effects associated with M_2 muscarinic receptor activation in all cardiac myocytes, especially in light of evidence supporting the idea that both types of responses may also be explained by direct regulation of AC activity. Some of the apparent discrepancies may be explained by species-dependent differences. However, in the future it will be necessary to address the question of whether or not there is developmental regulation of muscarinic signaling pathways *in vivo*, and whether or not there may even be differences in the signaling pathway mediating muscarinic inhibition and facilitation of cAMP-dependent responses in atrial and ventricular myocytes of a given species.

This work was supported by National Institutes of Health Heart, Lung, and Blood Institute Grant HL68170.

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(Received February 14, 2003
Revised April 7, 2003
Accepted April 29, 2003)