REVIEW

Muscarinic Receptors in Cardioprotection and Vascular Tone Regulation

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Summary
Muscarinic acetylcholine receptors are metabotropic G-protein coupled receptors. Muscarinic receptors in the cardiovascular system play a central role in its regulation. Particularly M_2 receptors slow down the heart rate by reducing the impulse conductivity through the atrioventricular node. In general, activation of muscarinic receptors has sedative effects on the cardiovascular system, including vasodilation, negative chronotropic and inotropic effects on the heart, and cardioprotective effects, including antifibrillatory effects. First, we review the signaling of individual subtypes of muscarinic receptors and their involvement in the physiology and pathology of the cardiovascular system. Then we review age and disease-related changes in signaling via muscarinic receptors in the cardiovascular system. Finally, we review molecular mechanisms involved in cardioprotection mediated by muscarinic receptors leading to negative chronotropic and inotropic and antifibrillatory effects on heart and vasodilation, like activation of acetylcholine-gated inward-rectifier K^+-currents and endothelium-dependent and -independent vasodilation. We relate this knowledge with well-established cardioprotective treatments by vagal stimulation and muscarinic agonists. It is well known that estrogen exerts cardioprotective effects against atherosclerosis and ischemia-reperfusion injury. Recently, some sex hormones and neurosteroids have been shown to allosterically modulate muscarinic receptors. Thus, we outline possible treatment by steroid-based positive allosteric modulators of acetylcholine as a novel pharmacotherapeutic tactic.

Key words
Muscarinic receptors • Muscarinic agonists • Allosteric modulation • Cardiovascular system • Cardioprotection • Steroids

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Introduction

Muscarinic acetylcholine receptors are membrane proteins that belong to the super-family of G-protein coupled receptors (GPCRs) that transmit their signals into the cell through heterotrimeric G-proteins. Five different subtypes referred to as M_1–M_5 exist in mammals. Individual subtypes differ in second-messenger signaling depending on coupling with individual G-proteins.

The M_1, M_3 and M_5 receptors are excitatory subtypes preferentially activating the $G_{q/11}$ class of G-proteins [1]. The activation of α-subunits of these G-proteins leads to activation of phospholipase C (PLC). PLC, in turn, produces the second messengers, diacylglycerol and 1,4,5-inositol trisphosphate (IP_3), which activate protein kinase C (PKC) and mobilize intracellular calcium stores, respectively. PKC phosphorylates several target proteins. The Ca^{2+} influx causes a variety of cascades of intracellular activity. The M_2 and M_4 receptors are inhibitory preferentially coupling with the $G_{i/o}$ class of G-proteins. Activated $G_{i/o}$ α-subunits inhibit adenylyl cyclase (AC) leading to a reduction in the production of cAMP. Although the preferential coupling, their specificity is not absolute. Importantly, βγ-dimers released upon activation of $G_{i/o}$ G-proteins activate the inward rectifying potassium channel and inhibit calcium channels in the heart, leading to decrease in excitatory potential [2, 3].

Muscarinic receptors mediate a wide range of physiological functions in the central nervous system (CNS) and the effects of the parasympathetic nervous

system in the periphery. All regions of the mammalian heart are innervated by parasympathetic (vagal) nerves, although the supraventricular tissues are more densely innervated than the ventricles. Heart rate is largely controlled by the internal pacemaker activity. In the absence of any external stimuli, sinoatrial pacing maintains the human heart rate in the range of 60-100 beats per minute [4]. Increased vagal tone diminishes heart rate and inotropy by modulation of potassium and calcium currents in the sinoatrial node [5]. Vagal tone balances increased inotropy and heart rate mediated by G_s-coupled β_2- and β_1-adrenergic receptors activated by adrenaline released from sympathetic neurons upon a load or systemic noradrenaline upon stress. The increased activity of the sympathetic nervous system leads to increased force of muscular contractions that in turn increases the stroke volume as well as peripheral vasoconstriction leading to high blood pressure that in the long term may lead to heart failure [6].

Non-neuronal acetylcholine (ACh) activity was also reported [7]. For example, muscarinic receptors have been localized at the endothelial cells of blood vessels as well as other tissues that lack cholinergic innervation including myocytes [8]. Many pieces of evidence also suggest the role of non-neuronal ACh in the modulation of the cardiovascular system [9].

Muscarinic receptors in the cardiovascular system in health

Effects of ACh in the cardiovascular system depend on the cell type and subtype of muscarinic receptors. In the heart muscarinic receptors mediate a decrease in inotropy and chronotropy and modulate parameters of ionic currents [10,11]. Based on ligand binding and cloning studies, the M_2 subtype is considered the predominant muscarinic receptor in the mammalian heart [12,13] as well as vasculature [14]. Later studies revealed expressions of other muscarinic subtypes with variable distribution throughout heart regions [15].

M_2 receptors

It was demonstrated that M_2 mRNA represents more than 90 % of total muscarinic mRNAs in rat atria and in either ventricle. The concentration of M_2 mRNA in the atria is more than twice as high as in the ventricles [15]. Pharmacologic evidence indicates that functional responses of atrial and ventricular cardiomyocytes to ACh are primarily associated with the activation of M_2 receptors [16]. Stimulation of M_2 receptors influences cardiac ion channels function via modulation of different cellular pathways. However, some cardiac effects of muscarinic receptor stimulation remain enigmatic.

In supraventricular (sinoatrial, atrial, and atrioventricular) myocytes, M_2 receptors positively modulate inwardly rectifying potassium channels via a membrane-delimited mechanism involving direct activation by the βγ-subunits released from the $G_{i/o}$ inhibitory G proteins (Fig. 1), resulting in hyperpolarization, thus slowing the heart rate by reducing the impulse conductivity through the atrioventricular node [16]. ACh also inhibits AC via M_2 receptors and $G_{i/o}$ α-subunits, resulting in a decrease in the production of cAMP.

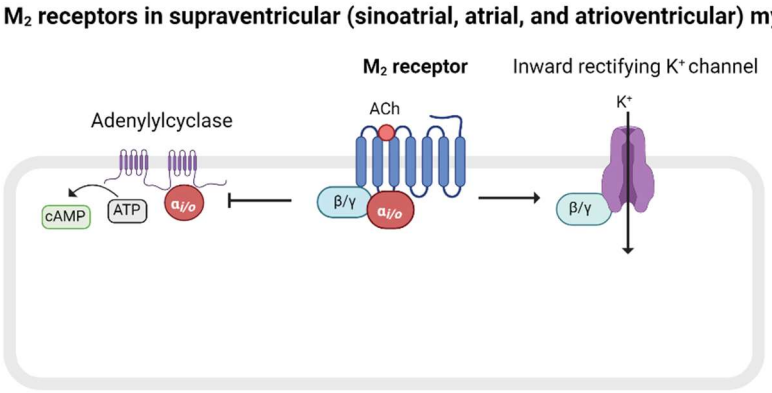

Fig. 1. M_2 receptor signaling in supraventricular myocytes. In supraventricular myocytes, M_2 receptors positively modulate inwardly rectifying potassium channels by the βγ-subunits released from the $G_{i/o}$ inhibitory G proteins resulting in hyperpolarization. "Created with BioRender.com."

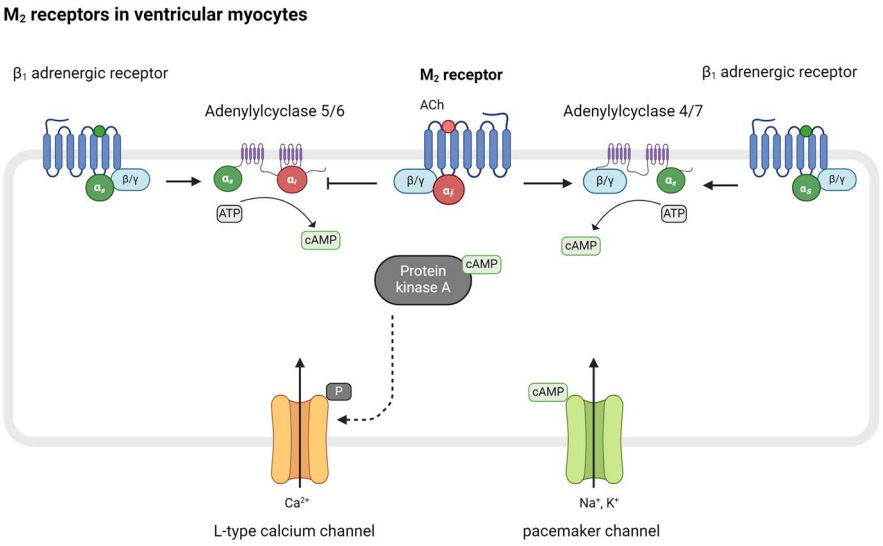

Fig. 2. M_2 receptor signaling in ventricular myocytes. In ventricular myocytes, M_2 receptors modulate the cAMP-dependent response to β-adrenergic receptor activation modulating activity of L-type Ca^{2+} and Na^+/K^+ pacemaker channels. "Created with BioRender.com."

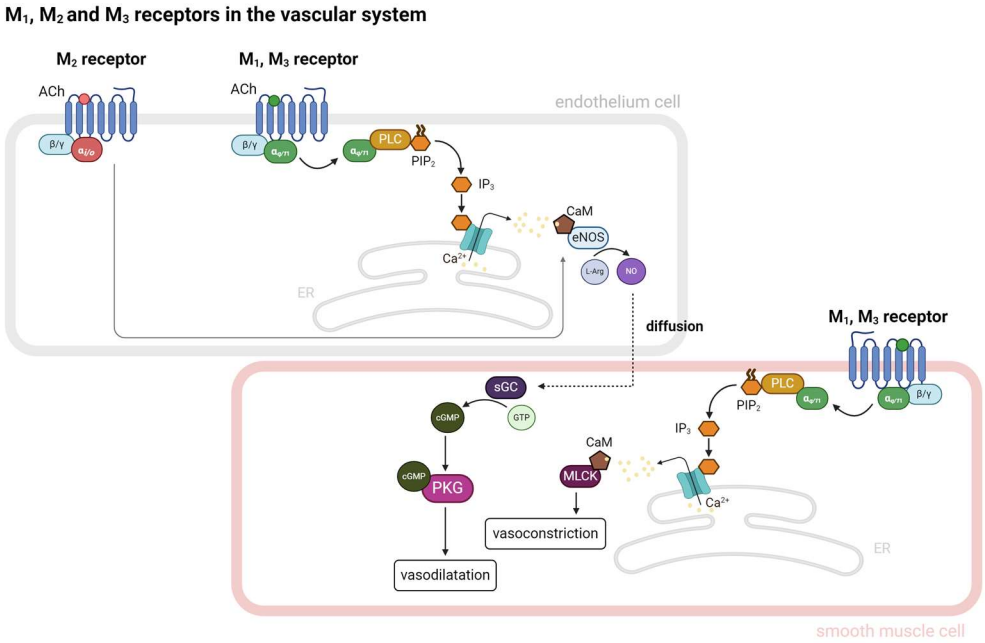

Fig. 3. Signaling of M_1, M_2 and M_3 receptors in the vascular system. In the vascular system, M_1, M_2 and M_3 receptors mediate vasodilation by release of NO from endotelium and cGMP-dependent removal of cytosolic calcium in smooth muscle cells, inhibiting the contractile apparatus and promoting vasodilation. "Created with BioRender.com."

In ventricular myocytes stimulation of the M_2 receptor has been shown to modulate the cAMP-dependent response to β-adrenergic receptor activation (Fig. 2) [17]. Responses to activation of M_2 receptors are only observed in the presence of agonists that stimulate cAMP production. The activation of M_2 receptors inhibits AC5/6 via the $G_{i/o}$ α-subunits and stimulates AC4/7 via the βγ-subunits released from inhibitory $G_{i/o}$ G-proteins. Changes in cAMP affect targets of protein kinase A (PKA)-dependent phosphorylation such as L-type calcium channels. Altering L-type calcium channel activity plays an important role in the regulation of cardiac myocyte contractility. Changes in cAMP also directly regulate pacemaker channels, which are permeable to both Na^+ and K^+ [16]. Moreover, it was demonstrated that presynaptic M_2 receptors inhibit the release of sympathetic noradrenaline in mouse atria, effectively cross-regulating sympathetic tone [18].

In the vascular system, the muscarinic M_2 receptors are located specifically in the endothelium of the

coronary vasculature from where they mediate endothelium-dependent vasodilation (Fig. 3) [19]. That is enacted through the release of NO from endothelium cells [20]. NO diffuses into the smooth muscle cell where it activates soluble guanylyl cyclase which converts GTP to cGMP. Cyclic GMP then activates cGMP-dependent protein kinase G, which results in the removal of cytosolic calcium, inhibiting the contractile apparatus and promoting vasodilation [21].

M_1 receptors

M_1 Muscarinic receptors are predominantly expressed in the CNS where they are involved in learning and memory [1]. However, the M_1 receptor has also been localized in numerous peripheral regions, including the cardiovascular system [22]. In the heart, the M_1 receptor is found in the cardiomyocytes of the human atria and ventricles [17,23]. The ACh gating of the inward-rectifier K+ channel is the main electrophysiological effector of vagal nerve stimulation in the atrium and contributes to atrial action potential duration shortening [24]. Although $G_{i/o}$ protein-coupled M_2 receptors are considered the predominant mediators of this process, it was shown that $G_{q/11}$-coupled M_1 receptors also participate in it (Fig. 4) [23].

Several experiments indicate that higher concentrations of muscarinic agonists than those needed for negative inotropy can induce opposite effects, including positive chronotropy and inotropy and prolonged duration of action potentials [11]. It was demonstrated using guinea pigs' ventricular cardiomyocytes that the M_1 receptors stimulate PLC and enhance the amplitude of the L-type calcium current, which may lead to a positive ionotropic effect, increase in heart rate and contractile force [25]. This effect was small and is elicited only by high concentrations of carbachol. The capability of the M_1 receptor to reverse depressed atrial contractility was demonstrated in the human myocardium [26]. It has been speculated that M_1 receptors in the human heart counterbalance the effects of M_2 receptors and might act as a limiting mechanism to prevent excessive suppression of cardiac activity [11].

The M_1 receptors have been reported to be distributed in the vasculature including arterial and venous smooth muscles and endothelium [27,28]. Overall, while stimulation of endothelial M_1 receptors leads to vasodilation, stimulation of M_1 receptors in smooth muscles leads to vasoconstriction. Many studies demonstrated that the M_1 receptors partially mediate ACh-induced vasodilation, which is dependent on the presence of an intact endothelium and the production of NO [14]. Moreover, it was demonstrated that M_1 may cause the endothelium-independent vasodilatation in rat mesenteric arteries that can be caused by the activation of M_1 receptors located on calcitonin gene-related peptide (CGRP)-containing neurons. The CGRP released from these neurons then acts at postsynaptic CGRP receptors on vascular smooth muscles causing the endothelium-independent vasodilation [28].

Although the principal effect of ACh in most vascular beds is endothelium-dependent vasodilatation, ACh can also produce direct vascular smooth muscle contraction. The M_1 receptors have been associated with vasoconstriction in several studies. For example, a study in guinea pig carotid arteries reported the M_1-mediated norepinephrine release, promoting constriction of the vascular tissue [29]. The dilation-constriction balance is influenced by vascular tone. In dilated arteries, the constrictor effect predominates. At high vascular tone, the vasodilatory response dominates [14].

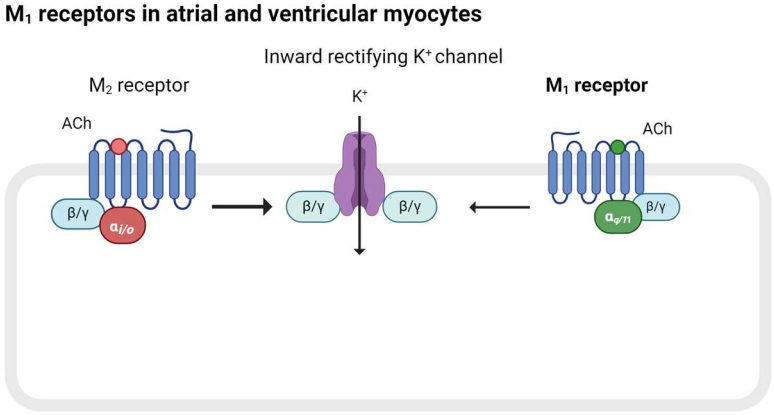

Fig. 4. Signaling of M_1 receptors in the heart. In the cardiomyocytes of the human heart M_1 receptors positively modulate inward-rectifier K^+ channel contributing to shortening of duration of atrial action potential duration. "Created with BioRender.com."

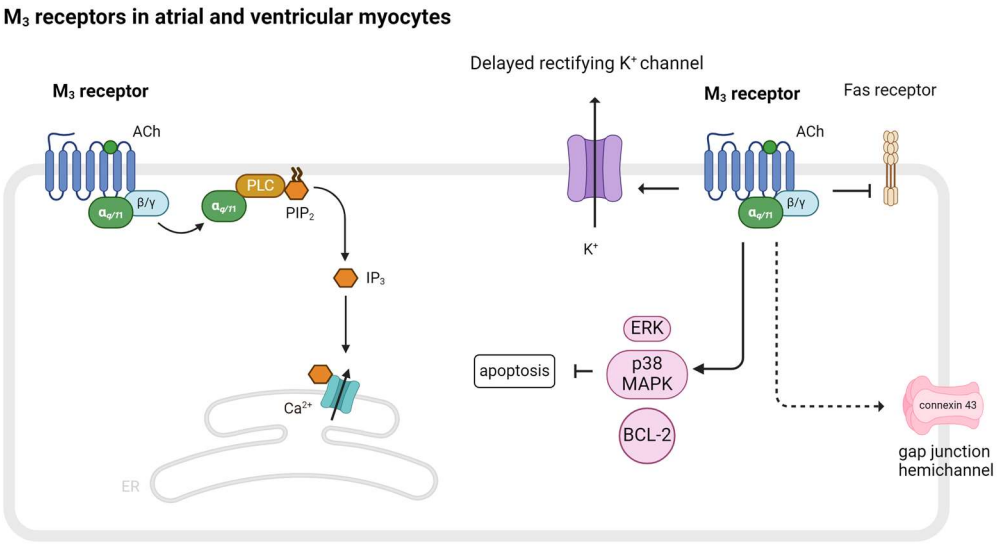

Fig. 5. Signaling of M_3 receptors in the heart. Through stimulation of intracellular phosphoinositide hydrolysis, M_3 receptors improve cardiac contraction. By the activation of a delayed rectifying K^+ current, M_3 receptors facilitate cardiac repolarization and exert negative chronotropic effects. "Created with BioRender.com."

M_3 receptors

The presence of muscarinic subtype M_3 was confirmed in both human atrial and ventricular tissues with 10-fold higher expression in ventricles [14]. Muscarinic M_3 receptors play an important role in the regulation and maintenance of cardiac function. M_3 receptors regulate intracellular phosphoinositide hydrolysis to improve cardiac contraction (Fig. 5). Through the activation of a delayed rectifying K^+ current M_3 receptors participate in cardiac repolarization and negative chronotropic actions. By activation of several antiapoptotic signaling molecules such as BCL-2 and ERKs, M_3 receptors enhance endogenous antioxidant capacity and reduce the apoptotic mediators (Fas and p38 MAPK) and Ca^{2+} overload resulting in cytoprotection [30,31]. Furthermore, M_3 receptors regulate the cell-to-cell communication via interaction with gap-junctional channel connexin 43. It was proposed that this interaction may be useful in coordinating the repolarization rates of the cardiomyocytes [32].

Muscarinic M_3 receptors are distributed throughout the vascular system in both endothelial cells and smooth muscle cells. Similarly to M_1 receptors, endothelial M_3 receptors mediate vasodilation and M_3 receptors in smooth muscles vasoconstriction. In several vascular beds, M_3 receptors have been shown to induce endothelium-dependent vasodilation, a process that, at least in part, is dependent on the release of NO [16]. It was suggested that in rat mesenteric arteries M_3 receptors mediate also endothelium-independent vasodilation [33]. Also in cats, activation of M_3 receptors in arteries with an intact endothelium leads to vasodilation, whereas activation of smooth muscles in the absence of endothelium induces vasoconstriction [34]. Furthermore, the experiments in bovine cerebral arteries reported M_3 receptor-mediated presynaptic inhibition of Ach and norepinephrine release, suggesting M_3 receptors regulate both vasoconstriction and vasodilation [35].

M_4 and M_5 receptors

Muscarinic receptors M_4 and M_5 appear to mediate the physiological function of ACh mainly in the central nervous system [1]. However, their presence was confirmed also in the cardiovascular system. Quantification of mRNA reported less than 1 % of the M_4 subtype and less than 5 % M_5 subtype of total muscarinic RNAs in the atria and ventricles [15]. Receptors that mediate muscarinic agonist-induced inhibition of high-voltage-activated Ca^{2+} channels in rat intracardiac neurons were identified as M_4 subtypes [36]. Although the function of $G_{i/o}$-coupled M_4 receptors overlaps with that of M_2 receptors, the role of M_4 facilitated bradycardia was not confirmed using M_4 knockout mice [37]. Some role for M_4 receptors in the control of K^+ channels was reported in canine atrial tissue [38]

RT-PCR experiments showed that M_5 receptor mRNA is present in several vascular tissues [39]. It was demonstrated that M_5 receptors mediate ACh-induced vasodilation in small cerebral blood vessels. A study on M_5 KO mice demonstrated that outside of the cerebral vessels, M_5 does not appear to play a significant role in vasodilation [40].

Muscarinic receptors in the cardiovascular system in aging and disease

After heart failure, an increased sympathetic tone may help to preserve cardiac function initially, it may also contribute to cardiac remodeling. Hyperactivity of the sympathetic nervous system is coupled with decreased activation of the parasympathetic nervous system observed early after induction of cardiac remodeling [41]. In patients with chronic myocardial infarction, muscarinic receptors are upregulated in remote non-damaged ventricular regions, indicating compensatory mechanisms to cardiac remodeling, while the receptor density remains within normal values in myocardial regions containing damaged tissue [42]. The expression of M_2 receptors is also elevated as a response to chronic pain leading to atrial fibrillation [43]. The density of muscarinic receptors as well as parasympathetic activity drop with age, thus, their cardioprotective as well as pharmacotherapeutic potentials decline [44,45]. The non-neuronal cholinergic system is also found in human cardiomyocytes, which express choline acetyltransferase and the vesicular acetylcholine transporter, enzymes necessary for ACh synthesis and release. Experiments on transgenic mice suggest that a compensatory increase in cardiomyocyte acetylcholine levels may help offset cardiac remodeling in heart failure [46].

Muscarinic receptors in cardioprotection

The activation of muscarinic receptors has primarily calming effects on the cardiovascular system, including negative chronotropic and inotropic effects on heart and vasodilation, suggesting that vagal stimulation or muscarinic agonists and positive allosteric modulators may have cardioprotective properties.

Indeed, vagal stimulation performed intermittently antagonized the sympathetic system and reduced the infarct size [47]. Activation of efferent parasympathetic nerves is involved in remote ischemic preconditioning that was lost after sectioning of the vagus nerves and after administration of atropine [48]. During ischemia-reperfusion injury, both continuous vagus nerve stimulation and intermittent vagus nerve stimulation provide significant cardioprotective effects. These beneficial effects were abolished by muscarinic blockade, suggesting the importance of muscarinic receptor modulation during vagus nerve stimulation. The protective effects of vagus nerve stimulation could be due to its protection of mitochondrial function during ischemia-reperfusion [49]. Thus, vagal stimulation may be an intervention used for treating cardiovascular diseases.

The beneficial effects of muscarinic agonists were observed very early. Cholinergic agonists and cyclic GMP can prevent ventricular fibrillation in susceptible animals independently of heart rate changes [50]. Furthermore, the muscarinic agonist oxotremorine reduced the incidence of malignant arrhythmias resulting from transient ischemia and sympathetic hyperactivity. The extent of this protection was comparable to the administration of β_2-adrenergic antagonist propranolol in this same animal model. The effect appears to be only partially dependent on the reduction in heart rate because it was observed even after the correction of heart rate by atrial pacing [51]. The antifibrillatory effects obtained without the marked reduction in myocardial contractility during acute ischemia, including administration of β-blockers in individuals with myocardial infarctions, are of special interest. Muscarinic agonists may therefore represent a new approach to the prevention of sudden cardiac death.

The data on cardioprotective effects of muscarinic agonists are overwhelming and provide detailed mechanistic views. Ischemia induces the production of the cytokine tumour necrosis factor-α (TNF-α) and reactive oxygen species (ROS) in a time-dependent manner. ACh is capable of inhibiting ischemia-induced TNF-α production, ROS generation, and cell death through the M_2 receptor and mitogen-activated protein kinases (MAPKs) [52]. The activation of M_2 receptors in *ex vivo* rat hearts leads to an increase in the activity of ERK1/2 and PI3K/Akt kinases that inhibit endoplasmic reticulum-induced stress leading to cell apoptosis that in turn contributes to a reduction in infarct size and attenuation of myocardial injury [53]. Moreover, ACh induced cytoprotective mitophagy by enhancing PINK1/Parkin translocation to mitochondria in an M_2 receptor-dependent manner in H9c2 cardiomyocytes [54].

Basal and acetylcholine-gated inward-rectifier K^+-currents ($I_{K,ACh}$), which play a significant role in atrial repolarization are upregulated in chronic atrial fibrillation. Not only M_2 receptors are involved in the cardioprotective effects of muscarinic agonists mediated by $I_{K,ACh}$. In human atrial cardiomyocytes, $G_{q/11}$ proteins activated by M_1-receptors act as donors of βγ-dimers that activate $I_{K,ACh}$. Their relative contribution to $I_{K,ACh}$ activation is increased in chronic atrial fibrillation patients [23].

M_3 receptors play a substantial role also in

cardioprotection, including regulation of heart rate and cardiac repolarization, modulation of inotropic effects, cytoprotection against ischaemic injuries of the myocardium, regulation of cell-to-cell communication, and generation and maintenance of atrial fibrillation. Signal transduction mechanisms underlying these functions involve activation of an $I_{K,ACh}$ participating in cardiac repolarization, negative chronotropic actions, and anti-arrhythmic as well as pro-arrhythmic actions, interaction with gap-junctional channel connexin 43 to maintain cell-cell communication and excitation propagation, regulation of intracellular phosphoinositide hydrolysis to improve cardiac contraction and haemodynamic function, activation of anti-apoptotic signaling molecules, enhancing endogenous antioxidant capacity, and diminishing intracellular Ca^{2+} overload, all of which contribute to protection of the heart against ischemic injuries [31].

Specifically, the overexpression of M_3 receptors reduced the incidence of arrhythmias and mortality after myocardial infarction and reperfusion by protecting the myocardium from ischemia in mice. This effect was mediated by increasing the $I_{K,ACh}$ currents by downregulation of expression of microRNA-1 (a single-stranded 22 nucleotides long noncoding RNA) that causes arrhythmia [55]. Also, the upregulation of M_3 receptors during myocardial hypertrophy could alleviate the hypertrophic response induced by angiotensin II by the mechanism involving the inhibition of MAPK signaling through the downregulation of the AT_1-angiotensin receptor [56]. Activation of M_3 receptors overall improves cardiac function and reduces ischemic myocardial injuries by multiple signaling pathways leading to cytoprotection [57], inhibits cardiac fibroblast proliferation and collagen secretion by TGF-β1/Smad and p38MAPK pathways [58], and exerts protective effects against ischemia-induced arrhythmias in the rat by a reduction of intracellular Ca^{2+} overload via downregulation of L-type Ca^{2+} channels [59]. Activation of M_3 receptors 24 hours before an ischemic insult induced delayed preconditioning in rats, preserving phosphorylated connexin 43, which might contribute to the anti-arrhythmic effect, and reduce the infarct size induced by infarction-reperfusion by up-regulation of cyclooxygenase-2 [60].

The alternative to vagal stimulation and muscarinic agonists in cardioprotection may be positive allosteric modulation of acetylcholine binding and/or functional response. Such compounds increase the cardioprotective effects of endogenous acetylcholine, primarily sympathetic tone but also non-neuronal acetylcholine. Among the major advantages of allosteric modulators are the conservation of the time-space pattern of signaling and defiance of overdose. Positive allosteric modulators of acetylcholine at M_2 receptor as well as other subtypes were identified [61–63]. A recent study has shown that phosphorylation of cardiac ryanodine receptor 2 by protein kinase G, contributing to the cardioprotective effects of cholinergic stimulation, may be enhanced by the allosteric modulator LY2119620 [64].

The sex hormone estrogen has received increased attention for its ability to exert cardioprotective effects against atherosclerosis, but it has become clear that estrogen also exerts a direct protective effect against ischemia-reperfusion injury on the myocardium [65]. Estrogen cardioprotective effects are due to rapid nongenomic actions. Estrogen binds to membrane estrogen receptors (mERs). The mERs are a group of receptors activating various signaling pathways including tyrosine kinases and protein kinases PI3K, Akt, MAPK, Src, PKA and PKC, and phospholipase C increasing the concentration of intracellular calcium [66]. Several steroid compounds positively affected heart function, exerting positive inotropic activity via Na^+,K^+ ATPase [67] or L-type Ca^{2+} channels [68]. In the search for novel steroid derivatives with biological activity on heart failure, two steroid derivates acting via M_2 receptors were synthesized [69]. Current research indicates that the fast non-genomic effects of steroids mediated by muscarinic receptors are due to their allosteric modulation of muscarinic receptors [70,71]. Thus, steroid-based positive allosteric modulators of muscarinic receptors, primarily the M_2 subtype, represent a novel class of cardioprotective compounds. However, this concept has been only little explored so far.

Conclusions

The cardinal role of muscarinic receptors in cardioprotection is well established. Along with that, muscarinic agonists and vagal stimulation are considered as viable cardioprotective treatments. In contrast, positive allosteric modulation of muscarinic receptors as an alternative or supplementary therapeutic approach is very little researched up to now, deserving attention and exploration.

Conflict of Interest

There is no conflict of interest.

Acknowledgements

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Abbreviations

ACh – acetylcholine; AC – adenylyl cyclase; CNS – central nervous system; $I_{K,ACh}$ - acetylcholine-gated inward-rectifier K^+-currents; IP_3 – 1,4,5-inositol trisphosphate; MAPK - mitogen-activated protein kinase; PKA – protein kinase A; PKC – protein kinase C; PLC – phospholipase C; ROS – reactive oxygen species

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