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MUSCARINIC REGULATION OF THE L-TYPE CALCIUM CURRENT IN ISOLATED CARDIAC MYOCYTES

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Summary

Muscarinic agonists regulate the L-type calcium current in isolated cardiac myocytes. The second messengers pathways involved in this regulation are discussed briefly, with particular emphasis on the involvement of cAMP and cGMP pathways.

Key Words: heart, cardiomyocyte, acetylcholine, isoprenaline, GTP-binding protein, adenylyl cyclase, cyclic AMP, cyclic GMP, nitric oxide, guanylyl cyclase, phosphodiesterase, calcium channel

Although intensively studied over the last decades, the cardiac effects of acetylcholine (ACh) are still not clearly described at the molecular level. In the whole heart, ACh exerts a direct negative chronotropic effect on the sino-atrial node, a negative dromotropic effect on atrio-ventricular conduction, a direct negative inotropic effect on the atria, and indirect negative inotropic and lusitropic effects which are observed both in atrial and ventricular tissues in the presence of a sympathetic tone (34,55,58). When used at concentrations >10 μM, muscarinic agonists exert an additional positive inotropic effect (34). In isolated cardiac myocytes, activation of the muscarinic receptors by ACh modifies the activity of second messenger pathways, ionic channels, contractile proteins and calcium homeostasis (34,55,58,91,92). These effects are attributed to the M2 receptors based on the use of selective ligands of the muscarinic receptors subtypes. However, functional M1 and M4 receptors are also expressed in cardiac myocytes of some animal species (23,91,101). The identity of the second messengers involved in the muscarinic regulation in cardiac myocytes is a field of controversy (34,47,58). To illustrate this purpose, we will briefly review the muscarinic regulation of the cardiac L-type calcium current.

The L type calcium current (I_{Ca}) is a major determinant of the plateau phase of the action potential and is the trigger of contraction in cardiac myocytes (see 34). Therefore, its regulation by sympathetic and parasympathetic systems and their respective neuromediators, noradrenaline and ACh, is most relevant for cardiac physiology. Since the muscarinic regulation of I_{Ca} consists essentially of an anti-adrenergic effect, it is necessary to first summarize the ß-adrenergic regulation of I_{Ca}. Beta-adrenergic agonists, such as isoprenaline, produce a stimulation of I_{Ca} in all types of cardiac myocytes studied so far (34,38). Activation of ß-adrenergic receptors stimulates the stimulatory GTP-binding protein (G_s protein), which in turn enhances cAMP production by adenylyl cyclase (18,20,34,38,58). The increase in cytoplasmic cAMP level activates the cAMP-dependent protein kinase (cA-PK) which is ultimately responsible for the increase in I_{Ca} (20,26,34,84). Similarly, adenylyl cyclase activation is involved in the stimulation of I_{Ca} by histamine (42,52), prostaglandin I_2 (2), parathyroid hormone (95,105,106), relaxin (30), serotonin (81), glucagon (66), VIP (100) and CGRP (74). Direct activation of adenylyl cyclase with forskolin

also leads to an increase in I_{Ca}, (3,19,35,37,75,82,83,109) which is not mediated by activation of a G protein (19,35,37,82, 83). Whether cAMP generation accounts for all the ß-adrenergic stimulation of I_{Ca} has been a matter of debate (38,108). However, most reports have now demonstrated that cA-PK inhibitors fully antagonize the ß-adrenergic stimulation of I_{Ca}, which confirms that cAMP is the only relevant second messenger involved in this regulation (38,82,83).

Muscarinic agonists reduce and/or eliminate the ß-adrenergic stimulation of I_{Ca} (14,36, 41,43). They also antagonize the stimulatory effects of histamine (58), prostaglandin I_2 (2), serotonin (81), glucagon (18), CGRP (74) and forskolin (37,38,51,75,80). In marked contrast, the stimulation of I_{Ca} by intracellular perfusion with exogenous cAMP or non hydrolyzable analogs of cAMP is not reduced by muscarinic agonists (9,14,29,75,80,82,83,85). This suggests that the locus of action of ACh is prior to cAMP generation. Besides, in microperfusion experiments, ACh was found to inhibit the isoprenaline-stimulated I_{Ca} only in the part of the cell superfused by both muscarinic and β-adrenergic agonists (45). This suggests that the inhibitory effect of ACh on I_{Ca} does not require a diffusible second messenger. The inhibitory effect of ACh (or carbachol, CCh) on I_{Ca} is prevented by pertussis toxin (9,75,80). The cardiac substrates of the toxin are the G proteins, G_o and G_i (20,34,97). G_i has been shown to antagonize the activation of adenylyl cyclase by G_s. The α_i subunit of the G_i proteins has been demonstrated to be the relevant subunit involved in the inhibition the type V and VI adenylyl cyclase, the cardiac isoforms. However, the muscarinic inhibition of I_{Ca} can be altered either by the selective inhibition of α_i (51) or by internal dialysis with $\beta\gamma$ complexes (9). Thus, the relative role of α_i and $\beta\gamma$ subunits in the muscarinic regulation of I_{Ca} is not fully understood. Surprisingly, in frog atrial myocytes, pertussis toxin injection antagonizes the effect of nanomolar ACh concentrations on I_{Ca}, but it does not fully abolish the inhibition of I_{Ca} by micromolar concentrations of ACh (56). This is observed even when the activation by ACh of the muscarinic potassium current, $I_{K,ACh}$ is totally suppressed by pertussis toxin. However, the pertussis toxin-resistant effect of ACh is still mediated by G proteins, since it is abolished by intracellular GDPßS. Intracellular application of non hydrolyzable GTP analogs (GTPγS and GppNHp) mimics, in an irreversible manner, the inhibitory effect of ACh on isoprenaline- or forskolin-stimulated I_{Ca} (19,75,80,82,83). Like ACh, these analogs were found to have no effect on the cAMP-stimulated I_{Ca}. Altogether, these data suggest that the muscarinic inhibition of I_{Ca} is due entirely to the inhibition of adenylyl cyclase.

In the absence of β-adrenergic stimulation, i.e. under « basal » conditions, ACh does not modulate I_{Ca} unless the adenylyl cyclase is under a tonic activation. This can be tested by investigating the effects of cA-PK inhibitors or phosphodiesterase (PDE) inhibitors on I_{Ca}. Indeed, if a basal activation of adenylyl cyclase is responsible for maintaining a baseline stimulation of I_{Ca}, then cA-PK inhibitors or PDE inhibitors will respectively inhibit or stimulate basal I_{Ca}. In the cells where such a behavior is found, ACh is also able to reduce the basal I_{Ca} (rabbit sino-atrial myocytes, 85), or to antagonize the stimulation of I_{Ca} by PDE inhibitors (ventricular myocytes, 54,72). This inhibitory effect of ACh is also pertussis toxin-sensitive (23,72,85) and, thus, likely results from a G_i-inhibition of basal adenylyl cyclase. What is the reason for the presence of a significant basal activation of adenylyl cyclase in some cells and not in others? Most likely the relative proportion of G_i vs. G_s, and/or of β-adrenergic vs. muscarinic receptors. Indeed, spontaneous activation of G_s and G_i by empty receptors has been unveiled in intact cardiac myocytes by the inverse agonistic effects of β-adrenergic and muscarinic receptors, respectively (44,63,64). Thus, ß-adrenergic antagonists, such as atenolol and propranolol (70), and muscarinic antagonists, such as atropine and AF-DX 116 (33), were found, respectively, to inhibit and stimulate I_{Ca} in the absence of any receptor agonist. Moreover, the spontaneous activation of $I_{K,ACh}$ by GppNHp was significantly slowed down by muscarinic antagonists (33). Therefore, the basal effect of ACh on I_{Ca}, like that of PDE- or cA-PK-inhibitors, may depend on which of the inhibitory G_i- and stimulatory G_s-pathways dominate at rest.

The results summarized above are consistent with the hypothesis that ACh inhibits I_{Ca} via a reduction of cAMP production. This conclusion is supported by some biochemical data (24,34,50,57,58), but not all. Other studies showed that ACh produces inhibitory effects on contraction and I_{Ca} without any detectable changes in cAMP levels or cA-PK activity (7,25,61, 76,77,87,111). In some of these studies, the effect of ACh was attributed to the stimulation of a phosphatase activity (1,25,40,57,76,77). Although this mechanism may participate in the overall effect of ACh, the lack of detectable changes in cAMP concentration at the cellular level does not necessarily exclude an inhibition of adenylyl cyclase as part of this effect (57). Indeed, cAMP changes may occur in discrete pools near the sarcolemmal membrane which would make them undetectable by classical means. Such local increases in cAMP, which lead to local elevations of I_{Ca}, have been shown to occur in isolated frog ventricular myocytes during the activation of β-adrenergic receptors (45,46). In these experiments, application of ACh to the part of the cell not exposed to the β-adrenergic agonist has no effect on I_{Ca}. Thus, activation of phosphatase activity can not alone provide a satisfactory mechanism for the muscarinic regulation of I_{Ca}.

In addition to the muscarinic inhibition of I_{Ca}, seen in the nanomolar to micromolar range of ACh concentration, higher concentrations of agonist produce a stimulation of I_{Ca}. (23,34). This stimulation occurs via a pertussis toxin insensitive pathway. Indeed, in guinea-pig ventricular myocytes, CCh enhances I_{Ca} after stimulation of the current by intracellular cAMP and this effect is increased after treatment of the myocytes with pertussis toxin (23). This stimulatory effect on I_{Ca} likely involves activation of M1 receptors, and may participate in the pirenzepine-sensitive increase in intracellular free calcium concentration and contraction induced by CCh (91, but see 92). The likely mechanism for this effect is activation of a pertussis toxin-insensitive G protein, leading to activation of phospholipase C, and activation of protein kinase C/IP_3 pathways.

More than twenty years ago, and in many studies since then, muscarinic agonists were shown to increase cGMP levels in the heart (21,34,58,59,61). The participation of this cyclic nucleotide in the effects of ACh on cardiac myocytes is still a matter of debate. One difficulty comes from the fact that a significant fraction of the cardiac cGMP production occurs in smooth muscle cells, where it participates in the relaxation of coronary vessels by ACh (22, 102). Cyclic GMP levels were also found to be raised by ACh or CCh in cell suspensions enriched with cardiomyocytes, suggesting that cGMP could play a role in the muscarinic regulation of cellular functions in these cells (11,47,49,59,61). A soluble guanylyl cyclase activating factor was found to be secreted by cardiac myocytes in the presence of muscarinic agonists (4). This factor could be nitric oxide (NO) since its production is abolished by treating the cells with L-arginine analogs, which are NO-synthase (NOS) inhibitors, and is restored by adding an excess of L-arginine, the natural NOS substrate (4). Furthermore, cardiac myocytes express the constitutive endothelial NOS (eNOS or NOS3), and can express the inducible NOS (iNOS or NOS2) under certain circumstances (4,5,13,47,89,90). However, in one study, the elevation of cGMP levels induced by ACh was also found to be insensitive to L-arginine analogs suggesting that factors other than NO may link ACh to cGMP production (94).

Since I_{Ca} is strongly regulated by the cGMP/NO pathway, it is tempting to speculate that this pathway participates in the muscarinic regulation of I_{Ca}. Intracellular application of cGMP or activation of soluble guanylyl cyclase activity by NO-donors produce stimulatory and/or inhibitory effects on I_{Ca} depending on the concentrations used and/or on the animal species (reviewed in 59). Stimulation of I_{Ca} may result from an increase in cAMP concentration consecutive to the inhibition by cGMP of PDE3, a cGMP-inhibited PDE (15,16,17,48,78,79, 95,103). Inhibition of I_{Ca} may involve either a reduction of cAMP level due to the activation by cGMP of PDE2, a cGMP-stimulated PDE (17,36,59,68,69,78,93,95), or the activation of the cGMP-dependent protein kinase (cG-PK) (27,53,59,79,96,98,99,103,104,107 but see 28). Modification of functional amino

acid residues may lead to additional effects on I_{Ca} with some NO-donors used at high concentrations (>100 μM) (8). Like ACh, the regulation of I_{Ca} by cGMP takes place only in the presence of an elevated intracellular cAMP concentration. However, unlike ACh, cGMP and NO-donors regulate I_{Ca} even when the adenylyl cyclase activity is bypassed by intracellular application of cAMP (15,17,36,53,59,68,69,79). The simplest explanation of this result is that ACh acts upstream and the cGMP/NO pathway downstream from cAMP generation. Thus, this pathway, if turned on by ACh, may represent a secondary mechanism of regulation that could play a modulatory role in the overall effect of ACh on I_{Ca}.

Surprisingly, in several recent studies performed in rabbit SA node (31,32) and AV node cells (29) and in rat ventricular myocytes (5), L-arginine analogs were found to fully antagonize the muscarinic inhibition of I_{Ca}, suggesting an obligatory role for NO in this regulation. In some of these studies, L-arginine could restore the effect of muscarinic agonists on I_{Ca} after application of the analogs (29,31,32). In cat atrial myocytes, application of a NOS inhibitor was shown to remove the rebound stimulation of I_{Ca} that follows ACh washout (107). Furthermore, methylene blue and LY 83583, two putative guanylyl cyclase inhibitors (10,73, 88), were shown to reduce the inhibition of I_{Ca} by ACh or CCh (5,29,31,32,54,72). While these studies would support the hypothesis that the activation of the NO/cGMP pathway takes a major part in the inhibitory effect of muscarinic agonists on cardiac I_{Ca} (reviewed in 47), several other studies do not. For instance, in frog ventricular myocytes, the inhibition of I_{Ca} by ACh is totally insensitive to L-arginine analogs in spite of the fact that cGMP and NO-donors produce clear effects on I_{Ca} (36,67-69). In guinea-pig ventricular myocytes, the muscarinic inhibition of the cAMP-stimulated chloride current is also totally resistant to L-arginine analogs (110). Furthermore, the results of the experiments with methylene blue should be interpreted with caution since this compound inhibits the muscarinic activation of $I_{K,ACh}$ which is clearly not mediated by the NO/cGMP pathway (32,43). A recent study of ours demonstrates that methylene blue acts as a muscarinic receptor antagonist (43). Moreover, LY 83583 and methylene blue are superoxide anion generators and, as such, can modify the redox equilibrium of the cells (6,60,62, see also 10,12,65,71). For instance, LY 83583 potentiates the stimulatory effect of isoprenaline on I_{Ca}, even in the absence of guanylyl cyclase activity (43,see also 110). Thus, at this stage, the issue of the participation of the NO/cGMP pathway in the regulation of I_{Ca} by ACh remains unsettled.

In summary, while inhibition of adenylyl cyclase was considered for many years as the only mechanism responsible for the muscarinic inhibition of cardiac I_{Ca}, several other mechanisms have come out recently, most particularly activation of phosphatase and activation of NO/cGMP pathway. However, the participation of these additional mechanisms varies greatly (from all to none) in different published studies. These discrepancies need to be clarified, and most particularly the issue of possible animal species and/or cardiac tissue differences. Other questions that will need to be examined include: i) the characterization of the mechanisms involved in the coupling of muscarinic M2 receptors to eNOS (pertussis toxin sensitive G protein? Ca_i?), ii) the potential role of NO in the muscarinic inhibition of adenylyl cyclase, iii) the respective roles of each mechanism (adenylyl cyclase, phosphatase, NOS) in the overall effect of ACh, and iv) the targets of NO and/or cGMP leading to ACh regulation of I_{Ca}.

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