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·Minireview·

Modulation of M4 muscarinic acetylcholine receptors by interacting proteins

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Abstract: Protein-protein interactions represent an important mechanism for posttranslational modifications of protein expression and function. In brain cells, surface-expressed and membrane-bound neurotransmitter receptors are common proteins that undergo dynamic protein-protein interactions between their intracellular domains and submembranous regulatory proteins. Recently, the $G\alpha_{i/o}$-coupled muscarinic M4 receptor (M4R) has been revealed to be one of these receptors. Through direct interaction with the intracellular loops or C-terminal tails of M4Rs, M4R interacting proteins (M4RIPs) vigorously regulate the efficacy of M4R signaling. A synapse-enriched protein kinase, Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), exemplifies a prototype model of M4RIPs, and is capable of binding to the second intracellular loop of M4Rs. Through an activity- and phosphorylation-dependent mechanism, CaMKII potentiates the M4R/$G\alpha_{i/o}$-mediated inhibition of M4R efficacy in inhibiting adenylyl cyclase and cAMP production. In striatal neurons where M4Rs are most abundantly expressed, M4RIPs dynamically control M4R activity to maintain a proper cholinergic tone in these neurons. This is critical for maintaining the acetylcholine-dopamine balance in the basal ganglia, which determines the behavioral responsiveness to dopamine stimulation by psychostimulants.

Keywords: striatum; Ca^{2+}/calmodulin-dependent protein kinase II; dopamine; kinase; phosphorylation; cocaine

1 Introduction

Muscarinic acetylcholine receptors (mAChRs) are a family of G protein-coupled receptors (GPCRs), and can be classified into 2 subcategories according to the subtype of G proteins they couple with: M1-like and M2-like groups. M1-like (M1, M3, and M5) receptors are linked with $G\alpha_q$ proteins. Activation of them stimulates phospholipase C to trigger a phosphoinositide-dependent signaling pathway. M2-like (M2 and M4) receptors are bound with $G\alpha_{i/o}$ proteins, and upon activation, they inhibit adenylyl cyclase and thereby decrease cAMP production and protein kinase A activity[1,2]. All mAChRs respond to the endogenous neurotransmitter acetylcholine and participate in the regulation of a variety of neuronal and synaptic activities[2].

Of the 5 mAChR subtypes, M4 receptors (M4Rs) exhibit several distinct characteristics. Firstly, M4Rs are most abundantly expressed in the striatum[3-5]. Secondly, within the striatum, M4Rs are preferentially co-expressed with dopamine D1 receptors (D1Rs) in striatonigral projection neurons, a population of medium spiny neurons that constitute the 'direct pathway' in the basal ganglia circuits[6,7]. Finally, M4Rs are concentrated in postsynaptic sites and can be found in the postsynaptic density microdomain, which enables these receptors to be sensitive to synaptic input changes and to play a pivotal role in regulating synaptic strength and effi-

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cacy[5]. Indeed, M4Rs vigorously regulate normal cellular and synaptic activities in the striatum. Malfunction of M4Rs is frequently associated with the pathogenesis of various mental illnesses, such as schizophrenia and substance addiction, and neurodegenerative disorders, such as Parkinson's and Alzheimer's diseases[8,9].

While M4Rs are involved in the regulation of many proteins, M4Rs themselves are the targets of many other regulatory proteins. The regulatory proteins, through a direct protein-protein interaction, modulate M4R expression and function in response to synaptic and intracellular signals. The present review summarized several M4R-interacting proteins (M4RIPs) identified so far, and their roles in modifying M4R activity and maintaining cholinergic homeostasis in the basal ganglia.

2 Interactions of M4R with non-enzymatic proteins

M4R is a member of the GPCR family. Like other GPCRs, each M4R subunit possesses several intracellular domains: 3 intracellular loops and one C-terminal tail. All parts of intracellular domains could harbor interactions with cytoplasmic or adjacent membrane-bound proteins. In particular, the third intracellular loop is much larger than the other intracellular domains, and provides an optimal region for protein associations. Currently, several proteins have been found to interact with intracellular domains of M4R.

2.1 Elongation factor and M4R In search for potential binding partners of M4R, McClatchy and co-workers found that elongation factor 1A2 (eEF1A2) interacts with the third intracellular loop *in vitro*[10,11]. This interaction is specific for M4Rs, because eEF1A2 does not interact with the third intracellular loop of M1 or M2 receptors. Native forms of M4Rs and eEF1A2 are also associated with each other in brain cells *in vivo*. The interaction between M4Rs and eEF1A2 may lead to a functional consequence. eEF1A2 has been found to inhibit M4R recovery to the cell surface after agonist-induced internalization[11]. Another isoform eEF1A1 shows the same regulation of M4R surface expression, although whether it can bind to M4Rs, just like eEF1A2, is unknown. eEF1A-induced regulation of surface expression seems to be specific for M4Rs, since eEF1A fails to affect surface expression of M1 receptor. Besides, the reverse regulation of eEF1A by M4Rs through an interacting mechanism may exist. eEF1A2 is a GTP-binding protein, essential for protein synthesis. Through the interaction with eEF1A2, M4Rs may drastically alter nucleotide exchange of eEF1A2, and probably in this manner, the receptor possesses the ability to regulate the rate of protein synthesis. Taken together, current data show a direct interaction between eEF1A2 and the third intracellular loop of M4Rs. This interaction paves the way for the 2 proteins to regulate functions of each other. However, further studies are needed to explore whether the rate of M4R-eEF1A2 interaction is regulated by some specific extra- or intracellular signals, and if so, the exact mechanism underlying this regulation should be revealed.

2.2 Endocytic adaptor protein 2 (AP2) and M4R The agonist-induced M4R internalization is an important way to decrease the receptor efficacy after its activation. In this type of homo-desensitization process, an endocytic pathway involving dynamin I and arrestin-2 is mainly responsible for M4R internalization. When dynamin I or arrestin-2 is functionally knocked out, synaptotagmin, a membrane-trafficking protein, can initiate 'rescue' and takes a full control of M4R internalization[12]. In the end, synaptotagmin, AP2, and M4Rs are suggested to form a ternary complex[12], in which the M4R C-terminal motif "YRNI" is supposed to be a core sequence to interact with AP2. However, this M4R-AP2 interaction needs to be experimentally proven. A further question concerning the potential M4R-AP2 interaction is the AP2 subunit specificity. AP2, an adaptor in the clathrin-dependent receptor internalization, consists of α, β, μ, and σ subunits[13]. It will be interesting to clarify which subunit is involved in M4R internalization.

2.3 Exocytic proteins and M4R Presynaptic M2 receptors and to a lesser extent M4Rs, interact with proteins of the exocytic apparatus, i.e., syntaxin and synaptosomal-associated protein of 25 kDa (SNAP-25) in rat synaptosomal tissue[14,15]. Since these interactions were assessed using coimmunoprecipitation, it is unclear whether M2/M4 receptors interact directly or indirectly with those proteins. Of note, the interaction between the receptor and exocytic proteins is

voltage-dependent, since the interaction is maximal at resting membrane potential, whereas disassociation occurs at higher depolarization. Functionally, the depolarization-dependent release of the exocytic proteins from the receptor allows engagement of these proteins in vesicle fusion and neurotransmitter release.

2.4 M4R-M4R dimerization Emerging evidence suggests that GPCRs can form dimers or even higher-order oligomers. This dimerization or oligomerization contributes to the regulation of the efficacy of receptor signaling and/or the selectivity of G protein coupling. mAChRs are among the GPCRs that dimerize or oligomerize[16]. In addition to M2 and M3 receptors, M4Rs are also found to be present on the cell membrane as dimers[17]. However, in the cell, the exact place of dimerization/oligomerization of M4Rs is unknown. Presumably, the assembly may occur in the endoplasmic reticulum and/or at the cell surface. As to crosslinking mechanisms, dimerization and oligomerization may in principle result from either covalent or non-covalent (ionic or hydrophobic) associations of M4R monomers. These associations may involve specific amino acids in defined extracellular or intracellular domains of M4Rs, although the precise amino acid sites have not been analyzed. At present, whether M4Rs heterodimerize with other muscarinic receptor subtypes or even with other GPCRs, and the functional implications of M4R dimerization and oligomerization are intriguing topics for future studies.

3 Interactions of M4R with enzymatic proteins

3.1 Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and M4R Apart from non-enzymatic binding partners, protein kinases are a new category of interacting partners of M4Rs. CaMKII has been recently identified to be one of these protein kinases. CaMKII binds directly to the second but not the third intracellular loop of M4Rs[18]. Besides, this binding is exclusive for both proteins, because M4Rs do not bind to CaMKIV and CaMKII does not bind to the second loop of other muscarinic receptor subtypes (M1, M2, M3, and M5). Of note, the interaction is sensitive to Ca^{2+} signals. The interaction rate can be significantly upregulated by the increase in cytosolic Ca^{2+} level. As a serine/threonine protein kinase, CaMKII usually takes its binding partners as phosphorylation substrates. In fact, CaMKII phosphorylates M4Rs at a specific threonine site within its binding region in the second loop of the receptor. CaMKII is a synapse-enriched kinase. Its interactions with M4Rs may have a significant impact on synaptic M4R function. In striatal neurons, CaMKII-M4R interactions could enhance the M4R-mediated inhibition of adenylyl cyclase and cAMP production. Behaviorally, CaMKII regulates behavioral sensitivity to dopamine stimulation. Acetylcholine in the striatum is known to antagonize dopaminergic transmission to maintain a proper acetylcholine-dopamine balance[19]. CaMKII can augment a cholinergic tone on M4Rs in response to dopamine stimulation, to limit or normalize motor responses to dopamine agonists. An interaction-dead peptide (Tat-fusion peptide) that disrupts the CaMKII-M4R interaction could therefore remove the synergistic influence of CaMKII on M4Rs and reduce the efficacy of M4Rs in suppressing dopamine stimulation. Thus, the Ca^{2+}-sensitive CaMKII-M4R interaction provides a new layer of molecular mechanisms for the regulation and maintenance of the acetylcholine-dopamine balance in the striatum. Given the importance of the acetylcholine-dopamine balance in the pathogenesis of neurological disorders derived from either dopamine overload (substance addiction) or dopamine depletion (Parkinson's disease), the CaMKII-M4R interaction model may give valuable hints for studying the causes of these disorders.

3.2 G protein-coupled receptor kinases (GRKs) and M4R Surface-expressed GPCRs are phosphorylated by not only second messenger-regulated kinases (although may not protein kinase A), such as CaMKII [16,20], but also members of the GRK family. In response to agonist stimulation, GRKs phosphorylate the intracellular domain of GPCRs, which causes the recruitment of β-arrestin from the cytosol. Arrestin-binding to the receptor prevents its coupling with the G protein and promotes its interaction with endocytic proteins to facilitate receptor internalization followed by receptor recycling. These transiently inducible events determine the receptor desensitization and resensitization following stimulation. In NG108-15 cells expressing predominantly the M4 subtype of muscarinic receptors, GRK2 activity is linked to the agonist-

stimulated internalization and desensitization of M4Rs[21]. Overexpression of GRK2 or dominant negative GRK2 can increase or reduce the rate of agonist-stimulated receptor endocytosis, respectively. Neither GRK2 nor dominant negative GRK2 has any effect on the rate of receptor recycling following internalization. In addition to GRK2, GRK4 and GRK5 may also regulate the internalization of M4Rs[22]. Thus, M4Rs, like many other types of GPCRs, are subject to the phosphorylation by GRKs, which at least contributes to the receptor desensitization following stimulation. However, at present, no experiments have been conducted to identify the GRK-dependent phosphorylation of M4Rs and the precise phosphorylation site(s) in response to agonist stimulation.

4 Conclusion

M4R is a member of the GPCR family. Like many GPCRs, it has a relatively larger intracellular domain, i.e., the third loop[23]. This loop provides a region for protein-protein interactions of M4R with a variety of M4RIPs. Through this interaction, M4RIPs regulate the dimerization and oligomerization, stability and turnover, externalization and internalization trafficking, phosphorylation and other post-translational modifications of the receptor, leading to changes in receptor function. However, currently, the protein-protein interaction between M4Rs and M4RIPs has not been fully elucidated. More M4RIPs are expected to be identified. Given the unique distribution of M4Rs in the striatum, specifically in the D1R-bearing striatonigral medium spiny neurons, the importance of M4Rs in maintaining a proper acetylcholine-dopamine balance in the basal ganglia should be emphasized. The roles of M4Rs in the pathogenesis of many psychiatric and neurodegenerative diseases deserve more attention and thorough investigation. A previous study has reported that CaMKII interacts directly with M4Rs in a Ca^{2+}-dependent manner[18]. By phosphorylating a selective amino acid residue, CaMKII regulates receptor function and receptor-mediated behaviors. Additionally, GRK-dependent phosphorylation of M4R is believed to actively regulate desensitization and resensitization of the receptor. In summary, studies on the interaction between M4RIPs and classical M4R are just at an infant stage. Extensive studies are needed to explore new enzymatic and non-enzymatic M4RIPs and to define their roles in controlling normal receptor expression and function and in the development of various neurological disorders.

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蛋白的相互作用调节 M4 乙酰胆碱受体的功能

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摘要：蛋白 - 蛋白间的相互作用可以调控蛋白的表达及其功能。在脑细胞中，胞内蛋白常与膜表面受体的胞内区域结合来调节受体的表达和功能。已有研究表明，Gαi/o 蛋白偶联的 M4 受体常受多种结合蛋白的调节。这些结合蛋白通过与受体位于胞浆的区域结合来调节M4受体信号通路的传导效率。钙离子/钙调蛋白依赖的蛋白激酶(Ca^{2+}/calmodulin-dependent protein kinase II, CaMKII)是近年来发现的一种主要密集于突触的蛋白激酶，与M4 受体的胞内第二个内环相结合。CaMKII 通过磷酸化 M4 受体来增强 M4 受体通路对环腺苷酶的抑制作用，进而减少 cAMP 的合成。在纹状体神经元中，M4 受体结合蛋白动态控制 M4 受体的活性，从而在这些细胞中维持适当的胆碱传导信号通路的活性。可控的胆碱信号通路在维持基底节神经元内胆碱与多巴胺信号的平衡中有至关重要的作用，并由此决定机体对精神兴奋剂引起的多巴胺刺激反应的灵敏度。

关键词：纹状体；钙离子 / 钙调蛋白依赖的蛋白激酶；多巴胺；激酶；磷酸化；可卡因