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COMMENTARY

THE MOLECULAR BASIS OF NEUROTRANSMISSION AT THE MUSCARINIC RECEPTOR

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Neurotransmitters, hormones, and many drugs cause their effects by interacting specifically with their receptors. In most cases these receptors are highly specialized proteins on the outside surface of the cell. For agonists but not antagonists these interactions may induce conformational changes in the receptor molecule. These receptor changes induced by agonists may affect the electrical properties of cells by changing the membrane permeability, which ultimately leads to a biological response.

The link between receptor activation and cellular response is mediated by various effectors. It has been hypothesized that receptors and effectors freely diffuse in the plane of the membrane and that coupling of the two takes place after the agonist–receptor complex has formed [1, 2]. Effectors may be ion channels or enzymes (e.g. adenylate cyclase). In the case where they are channels, receptor-mediated permeability changes can arise in two possible ways: (a) the channel effector directly couples to the receptor, and conformational changes in the receptor molecule induced by the agonist result in conformational changes in the effector, thereby altering its permeability; or (b) activation of the receptor indirectly leads to the change in permeability by activating some intracellular metabolic pathway that causes the change. Prolonged exposure of receptors to agonist (over-stimulation) may lead to changes in the receptor, the effector, or the receptor–effector coupling. The result is desensitization which is likely a necessary consequence of activation for many neurotransmitter receptors.

Different receptors can interact with common effectors and would therefore cause identical changes within the cell when these receptors are activated. In addition, the same receptor could be linked to different effectors on different cell types and, therefore, upon activation cause changes peculiar to the cell type. It is possible that some receptors for a neurotransmitter lack effectors on certain cell types, and in this case the receptors would be nonfunctioning.

The muscarinic acetylcholine receptor and assays for this receptor

Acetylcholine, a neurotransmitter in the peripheral and central nervous system, interacts with two kinds of receptors: muscarinic and nicotinic, a classification based upon the specificity and relative potencies of a series of agonists and antagonists. In the central nervous system, most of the cholinergic receptors are of the muscarinic type [3], and these may exist pre- or postsynaptically. The presynaptic receptors may be autoreceptors regulating acetylcholine release from the nerve terminals [4–6].

Radioligand binding techniques have been used to study the muscarinic receptor and have yielded much information on these receptors. From these studies we know, for example, that muscarinic receptors are generally of highest density in the extrapyramidal areas of the brain [7, 8], and that there is a single class of binding sites for muscarinic antagonists and multiple classes of binding sites for muscarinic agonists [9]. In addition, agonist and antagonist binding to muscarinic receptors are differentially affected by different agents (e.g. sodium ions and guanyl nucleotides) [10–13].

However, radioligand binding studies in general fall short of answering vital questions about how neurotransmitter receptors are involved in mediating a biological response. Biological assays of receptor activation are essential to learn about neurotransmission, and ideally such studies should be complemented with radioligand binding studies.

Contractions of pupils or of smooth muscle; stimulation of sweat, gastric or salivary glands; and complex cardiovascular events are among the most marked effects of muscarinic agonists [14]. However, most of the classical pharmacological studies of muscarinic receptors were based on measurements of smooth muscle contraction, or reduction of blood pressure [15–17]. More recently, measurements of changes in membrane potential and in membrane resistance of neurons upon iontophoresis of muscarinic agonists [18] and iontophoretic studies of smooth muscle cells [19, 20] have been electrophysiological techniques which have provided much of the early information on localization of muscarinic receptors. Most recently, muscarinic receptor-mediated cyclic GMP formation has been used as a biological assay for these receptors.

Molecular events associated with muscarinic receptor activation

At the molecular level, increased cyclic GMP formation is a muscarinic receptor-mediated response in the central and peripheral nervous system [21, 22].

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Of all the systems tested, a particular clone of cultured mouse neuroblastoma cells (clone N1E-115) shows one of the highest cyclic GMP responses (up to ~200-fold) upon muscarinic receptor activation [23, 24]. Therefore, these cells have been very useful for investigating mechanisms regulating this function of the muscarinic receptor. This cyclic GMP response in mouse neuroblastoma cells is rapid, transient (peaks at 30 sec and returns to basal levels after 2–3 min) [25], calcium-dependent [24], temperature-dependent [26], and occurs as well when histamine H_1 receptors on these cells are activated [27].

Why these or any other cells should synthesize such quantities of cyclic GMP upon receptor activation remains quite uncertain. Nonetheless, this cyclic nucleotide response represents a handy way of assaying for both muscarinic acetylcholine and histamine H_1 receptor function.

A role for cyclic GMP

Early studies regarding the possible functional role of cyclic GMP in the heart suggested that this cyclic nucleotide might mediate the negative inotropic [28] and electrophysiological effects [29] of acetylcholine. However, later reports from several laboratories have shown an apparent dissociation between the negative inotropic effects and the increases in cyclic GMP levels induced by cholinergic agonists [30, 31]. In addition, there was no correlation between cyclic GMP levels and muscarinic receptor-mediated electrophysiological effects in the atrium [32]. In some cases, agents that are known to elevate tissue cyclic GMP levels failed to mimic cholinergic effects [33]. On the other hand, it has been shown that iontophoretically applied cyclic GMP mimics electrophysiological effects induced by acetylcholine in mammalian neocortical neurons [34]. Most recently, when mouse neuroblastoma cells (clone N1E-115) were incubated with cyclic GMP, it produced hyperpolarization, an effect which was also produced by the muscarinic receptor agonist carbamylcholine [35].

These last results represent strong evidence for a role for cyclic GMP in mouse neuroblastoma cells. Yet, despite the abundant literature dealing with the effects or functions of cyclic GMP, there is still no consensus on a specific biological function for this cyclic nucleotide. Moreover, Murad *et al.* [36] have reported that in many cases correlation of the temporal effects of an agent on cyclic GMP accumulation with a given process are insufficient to draw conclusions regarding possible cause and effect relationships. In addition, since cyclic GMP and possibly its analogues in sufficient concentrations can influence phosphodiesterase activity [37] and can also activate cyclic AMP-dependent protein kinase [38], studies with exogenous cyclic GMP must be cautiously interpreted.

An effector for the muscarinic receptor: calcium channels

How does the interaction of the muscarinic agonist with its receptor lead to this cyclic GMP response? To answer this question, we must focus on the effector and the agonist–receptor–effector interaction.

The effector for muscarinic receptor-mediated cyclic GMP formation appears to be calcium channels. The evidence in support of this hypothesis comes from many different studies in various tissues. Some of the evidence is more direct than others:

1. Extracellular calcium is required for the response [24].
2. Calcium channel blockers (e.g. verapamil, Mn^{2+}, Ni^{2+} [E. El-Fakahany and E. Richelson, manuscript in preparation], Co^{2+} [39], and La^{3+} [13]) antagonize receptor-mediated cyclic GMP synthesis.
3. Guanylate cyclase, the enzyme that synthesizes cyclic GMP from GTP, is largely soluble in these cells [40] and in many other cell types [22] and there is, therefore, little or no membrane-bound enzyme to directly couple to receptors.
4. This mouse neuroblastoma enzyme is stimulated by calcium ions [40].
5. Receptor-mediated cyclic GMP synthesis cannot be demonstrated in broken cell preparations [41].
6. With intact cells and in the presence of extracellular calcium ions, calcium ionophores stimulate cyclic GMP formation [42].
7. Muscarinic receptor stimulation increase calcium influx [43].

Studies with mouse neuroblastoma cells suggest that as for smooth muscle cells [44] there are two types of calcium channels on these cells: one opened by membrane depolarization or by depolarizing agents ("voltage-dependent"), and one opened as a consequence of receptor activation by agonists ("voltage-independent") [39]. These two types of channels have different sensitivities to calcium channel blockers, such as lanthanide ions [13, 45], cobalt and D600 [39].

Some compounds that are thought to interact only with receptors may also interact with their effectors. Thus, we have recently obtained evidence to suggest that 2-haloalkylamines (phenoxybenzamine and dibenamine) bind to muscarinic receptors and to receptor-operated calcium channels * and that the affinity of these compounds for the channels is greater than that for the receptors. Antagonism by 2-haloalkylamines of a common calcium channel effector could explain their ability to block a disparate group of receptor-mediated responses (muscarinic acetylcholine, α-adrenergic, histamine H_1, serotonin, dopamine, and opiate) as well as responses due to non-receptor stimulants such as calcium, potassium and barium.

The activation of muscarinic receptors in many different tissues results in enhanced phosphatidylinositol turnover, a reaction that also results from stimulation of α-adrenergic or histamine H_1 receptors [46]. All of these receptors appear to have some features in common: (a) they cause an increase in cell surface calcium permeability, and (b) they mediate an increase in cyclic GMP formation. During phosphatidylinositol turnover, a cycle of reactions occurs in which phosphatidylinositol is both broken down and resynthesized, while the glycerol backbone

* E. El-Fakahany and E. Richelson, unpublished data.

of the molecule is conserved throughout the process. These reactions may control receptor-coupled calcium channels [47].

Receptor–effector interactions

Muscarinic receptor activation can affect calcium channels, and agents binding to the channel (e.g. lanthanide ions) may affect the receptors that couple to them by altering the affinity and maximum binding capacity of the receptor site for ligands, and the efficiency of the coupling between the receptor and the channel. The multiple sites of different binding affinity for muscarinic or other agonists may result from different degrees of receptor–effector coupling [48]. Hence, the receptor–effector pair can be perturbed by interactions that affect either partner.

Lathanide ions, which have a higher affinity for calcium binding sites than calcium, were found in electron microscopic studies to bind to the cell membrane of mouse neuroblastoma cells (clone N1E-115) in the form of randomly distributed dense patches (E. El-Fakahany *et al.*, manuscript in preparation). With the use of X-ray probe microanalysis, we have been able to analyze further the binding of lanthanum to these cells and have found that no lanthanum penetrated cells and that agonists were more effective than antagonists at displacing this lanthanum binding.

Incubation of mouse neuroblastoma cells (clone N1E-115) with the lanthanides terbium, europium, neodymium or lanthanum results in a concentration-dependent reduction in the maximum cyclic GMP response to full agonists of the muscarinic receptor and an increase in the response to partial agonists [13]. These lanthanides, as well as Ca^{2+}, also cause a significant decrease in the EC_{50} of the agonist which is accompanied by a significant decrease in the binding affinity of a muscarinic antagonist both for mouse neuroblastoma cells and for rat brain homogenates [13]. However, these ions increase the maximum binding capacity of muscarinic receptors in the cells but not in the brain homogenates, a result that probably reflects the different degree of coupling between the receptor and the channel in intact cells as compared to homogenized tissue. We believe that these lanthanides increase the efficacy of agonists by interacting with Ca^{2+} binding sites on a factor that couples the receptor to the calcium channels and that the lanthanides reduce maximum cyclic GMP responses by interacting with the calcium channels.

Regulation of the sensitivity of muscarinic receptors

Receptor-mediated responses are regulated at both pre- and postsynaptic sites. Desensitization is a phenomenon that is an important example of a postsynaptic mechanism of the regulation of receptor function. This well-known pharmacological phenomenon is defined as a decreased responsiveness of a tissue to an agonist after prolonged exposure to the agonist. For many receptors including muscarinic, desensitization is likely a direct consequence of receptor activation. Although the physiological significance of this subsensitivity is uncertain, it is probably a mechanism to avoid overstimulation of cells by agonists. Receptor alterations in relation to the development of drug tolerance relate to this phenomenon of desensitization as well [49].

Desensitization can be specific or nonspecific, that is, affecting one or several receptors on a cell. The first report of nonspecific desensitization was made by Cantoni and Eastman [50] who found that preincubation of guinea pig ileum with high concentrations of acetylcholine or histamine abolished the contractile response to a previously effective dose of either agonist. Specific desensitization was first observed by Barsoum and Gaddum [51] who showed that, after preincubation of fowl rectal caecum with histamine, this tissue became insensitive to histamine while the response to acetylcholine as well as other agonists was only slightly reduced. Subsequently, specific desensitization has been demonstrated for α- and β-adrenergic receptors [52–54], nicotinic acetylcholine receptors [55], insulin receptors [56], thyroid-stimulating hormone receptors [57], muscarinic acetylcholine receptors [58–62], histamine H_1 receptors [63], histamine H_2 receptors [64] and prostaglandin receptors [65].

The desensitization of β-adrenoceptor-stimulated cyclic AMP formation has been widely studied, and several mechanisms have been suggested for this phenomenon in different biological systems, such as a decrease in the number of β-adrenergic receptors [66–68] (possibly due to an internalization process [69]). However, steps following receptor activation could also be involved in this desensitization. For example, subsensitivity may be caused by increased activity of phosphodiesterase [70], or the uncoupling of the receptors and adenylate cyclase [71]. Desensitization of muscarinic receptor-mediated responses has not been studied as extensively as that for the β-receptor, although it has evolved recently as a matter of importance concerning regulation of cholinergic responses. Brodeur and Dubois [72] were the first to report the development of tolerance to some anticholinesterase agents, and they suggested that this could be due to subsensitivity of the muscarinic receptors. Recently, there have been several reports showing that there is a significant decrease in brain muscarinic cholinergic receptor binding sites following chronic treatment with these agents [59–62]. However, in the majority of studies, there was no correlation of receptor loss with a decrease in pharmacological activity. This criticism also applies to studies by Klein *et al.* [73] and Siman and Klein [74], who showed that incubation of mouse neuroblastoma-rat glioma hybrid clone NG108-15 and mouse neuroblastoma clone N1E-115 with high concentrations of muscarinic agonists results in loss of receptor sites.

In our laboratory we have been able to distinguish between receptor disappearance ("down-regulation") and desensitization of muscarinic responses. Richelson [58] has shown that incubation of mouse neuroblastoma N1E-115 cells with muscarinic receptor agonists for up to 30 min (short-term desensitization) results in a rapid and specific loss of cyclic GMP responses to the agonists with no change in the binding of a radiolabeled antagonist. The rate of this desensitization is dependent on the agonist efficacy and concentration. In addition, desensitization does not depend on cyclic GMP synthesis and

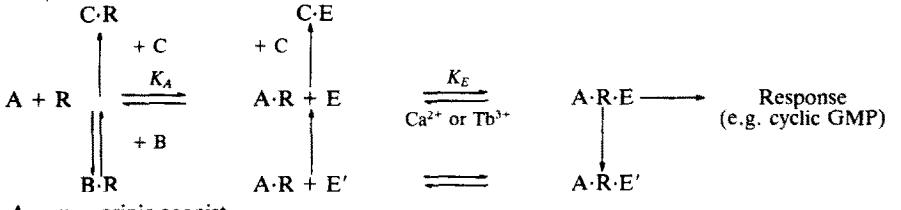
does not take place at temperatures below 20° [26]; this latter result suggests that the physical state of the membrane may influence this process. When the agonist is removed, there is a rapid return of sensitivity (resensitization) with a half-time of about 13 min [58]. The rate of resensitization is independent of the concentration or type of desensitizing agonist, suggesting that resensitization takes place after the agonist has dissociated from the receptor (L. Liu and E. Richelson, manuscript in preparation). Resensitization is also temperature-dependent, and the activation energies of desensitization and resensitization are 14 and 23 kcal · mol^{-1}, respectively [26], suggesting that protein conformational changes are involved in these processes.

Short-term desensitization of muscarinic receptor-mediated responses of mouse neuroblastoma cells is accompanied by no change in receptor number, receptor affinity for a radiolabeled antagonist, or guanylate cyclase activity (Ref. 58; E. El-Fakahany and E. Richelson, unpublished data). These data suggest that this rapid, specific desensitization results from a change in an agonist binding site of the receptor, or from an inactivation of the calcium channels that couple the receptor with guanylate cyclase, or a combination of both mechanisms. Recently, we have provided direct evidence that inactivation of calcium channels is the mechanism for this short-term desensitization [75].

In contrast to short-term desensitization, incubation of the cells with 1 mM carbamylcholine for more than 1 hr (long-term desensitization) results in a gradual decrease in the number and affinity of the muscarinic receptors as measured by radiolabeled antagonist binding, with a half-time for receptor disappearance of 4 hr [63]. After exposure of the cells to 1 mM carbamylcholine for 12 hr and then removing the agonist, there is a gradual recovery of the receptors, which is inhibited by protein synthesis inhibitors [63]. The return of receptor sites is much faster ($T_{\frac{1}{2}}$ = 6 hr) than the return of function of the muscarinic receptors ($T_{\frac{1}{2}}$ = 16 hr). Therefore, as with desensitization, there is a temporal dissociation between the appearance of muscarinic receptor function and the appearance of receptors.

Summary

The following hypothetical scheme represents a summary of the interactions considered here between the agonist (A), the muscarinic receptor (R), and the effector (E) (calcium channels in this case).



A = muscarinic agonist
B = "pure" muscarinic antagonist (e.g. atropine)
C = antagonist of the 2-haloalkylamine type
R = muscarinic receptor
E = active form of calcium channel effector
E' = desensitized effector
K_A = equilibrium dissociation constant for agonist–receptor complex
K_E = equilibrium dissociation constant for agonist–receptor–effector complex

This model involves the formation of a ternary complex between agonist–receptor complex and the effector and is in general, and in part, similar to what has been presented recently for the muscarinic receptor and for the β-adrenergic receptor–adenylate cyclase system. Results discussed here from studies on the muscarinic receptors of mouse neuroblastoma clone N1E-115 are compatible with this scheme.

In this model only agonists are capable of promoting the formation of the ternary complex, A·R·E, between the ligand, the receptor and the effector, while antagonists like atropine form only a binary complex with the receptor. Calcium and lanthanides act to promote the formation of the ternary complex involving the agonist, perhaps as allosteric activators. Partial agonists easily form the binary complex with the receptor but this binary complex has less affinity for the effector than the binary complex formed by full agonists except in the presence of an activator (high Ca^{2+} or lanthanides). Once the ternary complex is formed, the biological response occurs; next follows short-term desensitization which results from an inactivation of the effector. Resensitization involves the recovery of the effector from its desensitized state, E', to its active state E in conformance with a cyclic model (L. Liu and E. Richelson, manuscript in preparation). Antagonists of the 2-haloalkylamine type interact both at the receptor (R) and at the effector (E), but have higher affinity for E than for R.

Obviously, this is only a model and therefore requires further testing. The biochemical characterization of the effector will aid significantly in the clarification of our understanding of this system.

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