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COMMENTARY

MULTIPLE MUSCARINIC RECEPTORS IN THE CNS

SIGNIFICANCE AND PROSPECTS FOR FUTURE RESEARCH

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Muscarinic cholinergic receptors, which are located primarily on smooth muscle and in the CNS, appear to consist, at least on the basis of molecular weight, of only one or two peptides [1, 2]. The receptors are linked through GTP-binding proteins to second messenger systems that mediate the various cholinergic responses. The final common endpoint of receptor occupancy is the regulation of protein kinases, which have the capacity to alter various membrane conductances by the phosphorylation of specific proteins.

A dynamic multisubunit structure for receptors provides the opportunity for elaborate control over their characteristics and, thus, functions. This raises the possibility of "isoreceptors", similar to isoenzymes, which contain subunits coded by different genes, leading to distinct characteristics for each isotype. The possibility of receptor isotypes leads to a fundamental question: Do the apparent subtypes of muscarinic receptors represent different conformational states of the same entity, different peptides, or both? The isolation and characterization of muscarinic receptors are expected to shed light on this question.

Regardless of the molecular details, the existence of selective muscarinic ligands—both agonists and antagonists—has proven useful for a functional understanding of muscarinic receptors and holds exciting promise for the eventual pharmacologic treatment of neurologic disorders involving cholinergic deficits. The profound psychotomimetic effects of the potent anticholinergics in man [3, 4] and the involvement of cholinergic neurons in both Alzheimer's [5–8] and Parkinson's disease [9], together with observed behavioral impairments in animals after lesions of cholinergic pathways [10], attest to the importance of the central cholinergic system for memory and cognition as well as movement. In addition, muscarinic receptors in other brain areas serve major functional roles, such as the regulation of arterial pressure by the hypothalamus and brainstem [11].

Regional distribution of receptor subtypes

Subtypes of mucarinic receptors have been defined by several different criteria, each having advantages and limitations. Characteristics of associated ionic conductances [12], links to specific second messenger systems [13, 14] and preferences for various ligands [15, 16] have all been employed to explore the heterogeneity of muscarinic receptors.

Distinguishing forebrain from brainstem receptors. The overall picture that has emerged from binding studies—both with radioreceptor assays of homogenates and autoradiographic assays of sections—that address the regional differences in brain muscarinic receptors shows a clear distinction between receptors in the forebrain compared with those in the brainstem. In addition to differences in the patterns of the binding of selective ligands, the two regions can be distinguished on the bases of interactions with sulfhydryl reagents [17, 18], transition metal ions [18, 19] and guanine nucleotides [20–23].

Copper-deficient rats. Because Cu^{2+} displayed effects on agonist binding to muscarinic receptors at a concentration as low as 1 μM, well within the endogenous levels of Cu^{2+} in brain [19], muscarinic receptors were examined in the brains of rats made Cu^{2+}-deficient, as confirmed by atomic absorption spectrophotometry, on a dietary regimen [24]. Minimally deficient animals showed dramatic decreases in the binding of carbachol to forebrain regions, compared with control animals fed the same diet supplemented with Cu^{2+}, increasing IC_{50} values and derived dissociation constants. The effects on agonist binding could be reversed by adding Cu^{2+} exogenously to the binding assay *in vitro*. These findings suggest a role for Cu^{2+} in the endogenous regulation of muscarinic receptors in the forebrain.

Binding studies with homogenates. Even within brainstem and forebrain, or substructures grossly dissected from each area, the binding of agonists was found to be heterogeneous, providing the basis for one of the classification schemes. Analysis of the binding of carbachol, measured indirectly by its ability to compete for the binding of tritiated antagonists, suggested the presence of two or perhaps three binding sites—designated high (H), low (L) and super high (SH)—within the brain [25]. Subsequently, it was demonstrated that pirenzepine [15, 26] and gallamine [16] displayed heterogeneous binding within specific brain areas, with the binding of the former engendering the classification of muscarinic receptors as M_1 and M_2, designating receptors that have high or low affinity for pirenzepine respectively.

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Quantitative autoradiography. More recent autoradiographic studies of ligand binding to muscarinic receptors in brain have confirmed and extended the results with homogenates. Indeed, the brainstem and forebrain possess receptors with predominately high affinities for gallamine [27] and pirenzepine [28, 29] with the superior colliculus and the dentate gyrus representing extreme examples respectively. Moreover, autoradiographic technique permits the identification of exceptional areas such as the septum, which is a forebrain structure having a relatively high affinity for gallamine [27]. Two somewhat different approaches to the mapping of receptor subtypes by autoradiography have been used. In the first, the distributions of high-affinity sites for a number of tritiated selective ligands were compared [29]. Although the method is free of possible complications and artifacts that may be introduced by competitive assays, direct quantitative comparisons are difficult due to several factors, including uncertainties in the absolute values of specific activities and the chemical purities of labeled ligands and the possibilities of differential penetration and other interactions with the tissue sections. Some of these factors are eliminated by indirect assays, comparing the abilities of various ligands, several of which can be used in the same assay, to compete for the binding of a tritiated antagonist such as [^{3}H]quinuclidinyl benzilate ([^{3}H]QNB) which has a high affinity for all receptors.

Agonists. As labeled agonists became available, they were used to map the regional distribution of muscarinic receptor subtypes. The utility of tritiated agonists for this purpose is limited, however, by their inability to label all sites as well as the possible complications that may arise from the agonist-induced coupling of receptors to their effector systems.

Recently, Gurwitz and his associates [30] used the tritiated transmitter itself, [^{3}H]acetylcholine, in the presence of the cholinesterase inhibitor diisopropyl fluorophosphate to examine muscarinic receptors with high affinity for acetylcholine in rat cortical membranes. The data confirmed the result with competition experiments that receptors with high affinity for typical agonists represent only a minor proportion of muscarinic sites in the cortex. In addition, an increase in the number of high-affinity sites as a result of treatment with transition metal ions could be demonstrated directly, the effect being reversible by guanine nucleotides.

The results of binding studies with the agonists [^{3}H]*cis*-methyldioxolane [31] and [^{3}H]oxotremorine-M [32] have shown that, similar to [^{3}H]acetylcholine, they also label fewer muscarinic receptors than the classical antagonists. There is some evidence to suggest that [^{3}H]oxotremorine-M at low concentrations labels M_2 muscarinic receptors selectively [33]. The ligand, which was found to be suitable for autoradiographic analysis, bound uniformly to sections of the rat inferior colliculus with an affinity in the low micromolar range.

Selective antagonists. The antagonist pirenzepine, the selectivity of which defines the M_1 subtype, has been used indirectly in unlabeled form [34] and as [^{3}H]pirenzepine [26, 35] to map the distribution of M_1 muscarinic receptors throughout the brain with comparable results, showing the preponderance of M_1 receptors in forebrain regions as mentioned above. On the other hand, the antagonist gallamine has been used only in indirect studies [27], probably owing to its relatively low affinity even for its "high-affinity" sites. The regional distribution—similar to that for carbachol but the opposite of pirenzepine—of sites having a high affinity for gallamine in the rat brain suggests that gallamine is selective for M_2 muscarinic receptors.

There has been some controversy with respect to the nature of the binding of gallamine to muscarinic receptors. Ellis and Hoss [16] first presented evidence suggesting that gallamine binds in a competitive manner to brain muscarinic receptors when measured by its ability to reduce the binding of the universal antagonist [^{3}H]-*l*-QNB to rat brain membranes. Subsequently, Stockton *et al.* [36] showed by kinetic measurements that the effect of gallamine on the binding of [^{3}H]*N*-methylscopolamine ([^{3}H]NMS) to both rat heart and brain membranes was allosteric, reducing both the on- and off-rates of [^{3}H]NMS in a differential manner. Although Ellis and Lenox [37] were able to corroborate the effects of gallamine on the binding of [^{3}H]NMS, no such effects of gallamine on the binding of [^{3}H]-*l*-QNB were observed. With the caveat that the appropriate conditions for observing allosteric effects of gallamine on the binding of [^{3}H]-*l*-QNB may not have been attained, a likely explanation of the results is that NMS and QNB see muscarinic receptors differently. Indeed, as discussed below, a one-to-one correspondence between receptors occupied by the two ligands is lacking.

The overall distribution of M_1 and M_2 muscarinic receptors together with the finding that chemical denervation of basal forebrain areas in rats produced a selective reduction in M_2 receptors in the cortex [38], suggested a post- and presynaptic locus for M_1 and M_2 receptors respectively. In another study [39], however, denervation of the nucleus basalis in rats produced a 14% loss of sites having low affinity for carbamylcholine (roughly corresponding to the M_1 subtype) at 72 hr and a 28% increase in sites having high affinity for agonists (roughly corresponding to the M_2 subtype) without changing the total number of receptors in the rat cortex after 5 weeks. The disparity between the two reports may reside in the method of lesion or the choice of ligands for measuring receptors.

The functional selectivity of both gallamine and pirenzepine is evident in the muscarinic cholinergic responses of the rabbit superior cervical ganglion measured electrophysiologically [12]. Whereas gallamine selectively suppresses the slow-inhibitory postsynaptic potential, pirenzepine selectively suppresses the slow-excitatory postsynaptic potential. These results suggest the existence of distinct muscarinic receptors coupled to separate effector systems in this preparation.

Classical antagonists. The antagonists [^{3}H]-*l*-QNB and [^{3}H]NMS, especially the latter in more recent times because of its faster kinetics and thus more rapid attainment of equilibrium, have been employed as the principal ligands for measurements

of muscarinic receptors. Two recent reports [40, 41] suggest, however, that NMS does not see all of the receptors identified by QNB. Since the same disparity is observed with the N-methylated derivatives of other classical antagonists including scopolamine, atropine and QNB itself, it is likely that the differences in total numbers of receptors identified by a particular classical antagonist is related to its tertiary or quaternary nature. In other words, quaternary derivatives of classical muscarinic antagonists distinguish between subtypes of muscarinic receptors with the same specificity as the quaternary atypical antagonist gallamine.

It is evident from the foregoing discussion that selective ligands fractionate muscarinic receptors into different assemblies of subsets, which, although overlapping, may not directly correspond to each other.

Second messengers

Activation of muscarinic receptors in brain as well as other tissues has been associated with a number of biochemical responses, including inhibition of adenylate cyclase [42], stimulation of cAMP-dependent phosphodiesterase [43], stimulation of guanylate cyclase [44–46] and stimulation of phosphoinositide turnover [13, 14]. The emerging idea that various GTP-binding proteins may couple muscarinic receptors to each of these and other as yet undefined second messenger systems concomitantly adds depth to, and helps direct, new research efforts aimed at the understanding of muscarinic cholinergic responses.

Regulation of cAMP levels. Muscarinic agonists inhibit the activity of the enzyme adenylate cyclase in both whole cells and cell-free homogenates of brain [42], as well as many other tissues, and cultured cell lines derived from neurons [47]. An additional mechanism for the receptor-mediated reduction in cAMP levels—activation of cAMP-dependent phosphodiesterase—has been observed with certain clonal cell lines [43]. With the astrocytoma line 1321N1 this activity is Ca^{2+}/calmodulin dependent and thus may be linked to phosphoinositide metabolism discussed below [48, 49]. This picture is somewhat reminiscent of light-induced stimulation of cGMP-dependent phosphodiesterase, with its relationship to Ca^{2+} and mediation by the GTP-binding protein transducin, in the outer segments of retinal rod cells [50].

Similar to other hormone receptors linked in an inhibitory manner to adenylate cyclase, inhibition of the enzyme is GTP dependent [42, 51], and GTP (or its stable analogs) reduces the binding of agonists but not antagonists to the receptor [21]. A great deal of evidence now suggests that these effects are mediated through the GTP-binding protein G_i, a multisubunit protein complex that interacts with both inhibitory receptors and the catalytic unit of adenylate cyclase [52]. An important advance in this area has been the development by Murayama and Ui [53] of pertussis toxin, which catalyzes the ADP-dependent ribosylation of the alpha subunit of G_i, as a tool for its selective inactivation; thus, treatment with pertussis toxin eliminates the ability of muscarinic agonists to inhibit adenylate cyclase and reduces their affinity [54]. It had been discovered earlier that a similar protein, G_s, which couples hormone receptors linked in a positive manner to the catalytic unit of adenylate cyclase, could be inactivated by cholera toxin [55]. The specificities of the toxins are not absolute, since transducin, a distinct protein complex, is a substrate for both [56, 57]. Further, the inability of cholera or pertussis toxins to inhibit either the muscarinic agonist-induced activation of cAMP phosphodiesterase [58] or the binding of carbachol to muscarinic receptors on 1321N1 astrocytoma cells [59] suggests that phosphodiesterase is linked to muscarinic receptors via a GTP-binding protein distinct from G_s or G_i.

Cholera and pertussis toxins are not isolated examples, but rather members of the class of so-called A–B toxins including tetanus, botulinum and diptheria toxins, the last of which catalyzes the ADP-dependent ribosylation of elongation factor 2, resulting in the inhibition of protein synthesis [60]. The discovery of additional toxins that inactivate GTP-binding proteins other than G_s and G_i can be expected in the future.

Stimulation of GTPase. Apparently as a means of inactivating receptor–effector coupling, the alpha subunit of G_i, as well as other GTP-binding proteins, hydrolyzes bound GTP to GDP, creating by this GTPase activity a form of G_i that is incapable of the coupling process [61, 62]. Exchange of bound GDP for GTP then restores the ability of G-proteins to couple muscarinic receptors to their effectors [63].

Hormone-stimulated GTPase activity can be measured by utilizing gamma [^{32}P]GTP as a substrate for the enzyme [61], providing an additional index of receptor activation that is independent of the particular second messenger system and more proximal to receptor occupancy. Muscarinic agonists produce a saturable, concentration-dependent stimulation of low K_m GTP hydrolysis with synaptic membranes prepared from rat forebrain [64]. Both carbamylcholine and oxotremorine, which is more potent but less efficacious, stimulate GTPase in this preparation, having EC_{50} values of 5.8 and 0.16 μM respectively. Since, in the brain, oxotremorine has virtually no ability to stimulate phosphoinositide metabolism [65, 66] but is a full agonist for inhibiting adenylate cyclase [42], oxotremorine-stimulated GTPase activity may represent the component linked to adenylate cyclase. The Hill coefficients for the agonists are approximately 0.5, suggesting the presence of receptor multiplicity, negative cooperativity, or both. The selectivity of oxotremorine compared with carbamylcholine may be related to the difference between tertiary and quaternary amines.

Maximal carbachol-stimulated GTPase is inhibited by muscarinic antagonists in the order of potency atropine > pirenzepine > gallamine, consistent with the overall affinity profiles for these agonists in forebrain regions [64]. The Hill coefficients for the inhibition are approximately 0.4, further evidence for multiple receptors and/or negative cooperativity. These findings bolster the notion that multiple GTP-binding proteins are involved in muscarinic responses in the brain. The production of monoclonal antibodies to individual G-proteins associated with muscarinic responses will aid in the mapping of the

regional distribution of G-proteins, providing a comparison with known distributions of muscarinic receptor subtypes.

Stimulation of PI turnover. Muscarinic agonists have long been known to stimulate phosphoinositide (PI) turnover in brain [67, 68]. Receptor-mediated PI turnover can be assessed in brain slices by measuring released inositol phosphates after incorporation of [^{3}H]inositol to equilibrium with reincorporation of released products blocked by Li^+ [69]. Under these conditions, carbamylcholine stimulates PI turnover with a flattened dose–response, reaching a maximum value at approximately 1 mM.

The ability of selective muscarinic antagonists to inhibit PI turnover in rat striatal slices has been compared to the binding of the same ligands to muscarinic receptors under the same conditions [70]. Trihexyphenidyl, which has been reported to be selective for central versus peripheral muscarinic receptors [71], and pirenzepine were highly potent inhibitors of carbachol-stimulated PI turnover. Trihexyphenidyl was equipotent in occupying receptors and in inhibiting PI turnover. Pirenzepine was, however, much less potent at occupying receptors in the rat striatal slices, displaying nearly complete inhibition of PI turnover at 1 μM with only a small proportion of receptors occupied. Gallamine was much weaker than either of the others at both the inhibition of PI turnover and receptor occupancy. These findings may reflect the selectivity of pirenzepine for M_1 muscarinic receptors, which in the rat striatum appear to be linked to PI turnover. In contrast to these findings, pirenzepine was not selective for inhibiting the PI turnover response in embryonic chick heart cells, showing a higher potency for the adenylate cyclase response [72].

According to current concepts, activity in the PI cycle produces two different intracellular messengers—diacylglycerols, which activate protein kinase C, and inositol triphosphate, which releases Ca^{2+} from the endoplasmic reticulum [73]. Thus, muscarinic receptor-mediated stimulation of PI turnover may secondarily activate processes, such as guanylate cyclase and phosphodiesterase activities, which are sensitive to increased intracellular Ca^{2+}.

There is a striking overlap between the distribution of the binding of phorbol esters [74], which potently activate protein kinase C by occupying the diacylglycerol site [75], and muscarinic receptors in the rat forebrain (Hoss and Messer, unpublished observation). In other systems, protein kinase C produces a negative feedback on stimulated PI turnover by phosphorylating (and inhibiting) a protein (B-50) that catalyzes one of the steps in the PI turnover cycle [76]. Accordingly, phorbol myristate acetate prevents the muscarinic stimulation of PI turnover in embryonic chick heart cells [72].

The idea of the regulation of a PI response by a GTP-binding protein is not novel. Using pituitary-derived GH_4 cells, the thyrotropin-releasing hormone (TRH), which binds to specific receptors, stimulates PI turnover [77] and low K_m GTPase without affecting adenylate cyclase [78].

Possible relationships between receptors

The knowledge of whether the apparent heterogeneity of muscarinic receptors in the brain is a reflection of distinct receptor peptides, the identical binding unit coupled with multiple effectors, different conformational states of the same receptor, or some other factor, is dependent upon the characterization of pure isolated receptors. Further, it is expected that studies of isolated receptors will lead to a deeper understanding of the molecular nature of the receptor, its interaction with second messenger systems, and eventually to cloning of the c-DNA and complete sequence determination of the subunits.

Properties of receptors isolated from heart and brain. Although the solubilization of muscarinic receptors had been achieved several years ago in a number of laboratories [79–81], only recently has a suitable affinity ligand been developed. Purifications of muscarinic receptors from porcine atria [1] and cerebral cortex [2, 82] using 3-(2′-aminobenzhydryloxy)tropane linked to Sepharose have now been reported. Whereas the purified receptor from heart consists of an 80 kilodalton (kD) peptide that contains the binding site and a smaller peptide of 14 kD [1], the purified brain receptor has an apparent molecular weight of 70 kD with a lesser amount of the 14 kD species [2].

There is some evidence that the solubilization process itself affects the receptor subtype distribution for a given tissue. For the receptor from heart, which has been studied more extensively, solubilization converts receptors from high to low affinity for agonists [83]. Interestingly, digitonin-solubilized receptors from brain retain their response (conversion from low to high affinity for carbachol) to low concentrations of copper [84], suggesting that receptors with low affinity for agonists such as carbachol, roughly corresponding to the M_1 subtype, may be solubilized preferentially by these methods or that the solubilization process converts receptors from high to low affinity. It is also important to note that purified receptor reconstituted with GTP-binding protein displays a GTP effect on the binding of agonists [85].

Possible monomer–dimer equilibrium. Based on photoaffinity labeling experiments, the suggestion was made that brain muscarinic receptors can exist as a monomer or dimer of an 86 kD peptide [86], corresponding to low (M_1) and high (M_2) agonist affinity states of the receptor respectively. It is also possible based on other systems that the higher molecular weight species represented the receptor associated with a GTP-binding protein [87, 88].

Protection experiments. By inactivating certain subsets of receptors, for example those having low affinity for a particular ligand, it is possible to examine the remaining receptors in relative isolation. Experimentally this has been achieved by incubating receptors with unlabeled irreversible or very slowly reversible ligands in the presence of a selective reversible ligand, washing to remove the unbound and selective ligands, and subsequently examining the binding properties of remaining receptors. In general, such experiments have confirmed the heterogeneity of muscarinic receptors, that is, remaining receptors retain their original properties and do not spontaneously interconvert to the original mixture of subtypes [16, 89]. The selective protection of sites

having a high affinity for gallamine points to the usefulness of this ligand for distinguishing between receptor subtypes [37].

Link between muscarinic receptors and memory

Effects of central anticholinergics in man. The potent centrally-acting antimuscarinic agents such as QNB and its congeners are psychotomimetic and produce a long-lasting amnesia in man [3, 4]. Intoxication produces confusion, delirium and hallucinations, leading often to behavior that resembles an acute schizophrenic episode. Based on these observations and studies demonstrating memory deficits after the administration of scopolamine [90], it appears that central cholinergic pathways are important for human memory and cognitive function. Postmortem studies of Alzheimer's patients have revealed a loss of presynaptic cholinergic markers [5–7], loss of cells in the nucleus basalis [8], which is the major source of cholinergic neurons innervating the cortex, and subiculum [91], which contains the pathways that link the cortex and hippocampus. Neurons are also lost within the hippocampus [92], a structure known to be important in memory. Biochemically, it has been reported that M_2, but not M_1, receptors are lost in the cortex [38]. This important observation has led to the suggestion that M_1 agonists, by enhancing activation of postsynaptic receptors, or M_2 antagonists, by preventing the regulation of acetylcholine release presynaptically, may have therapeutic value in Alzheimer's disease. It is of interest in this regard that an oxotremorine analog, *N*-methyl-*N*-(1-methyl-4-pyrrolidino-2-butynyl)-acetamide, acts both as a presynaptic antagonist and a postsynaptic agonist [93].

Representational versus dispositional memory in animals. Memory has been divided into a number of overlapping dichotomous distinctions ranging from primary versus secondary [94] to working versus reference [95] memories. We have employed the distinction set forth by Thomas and his colleagues [10, 96] of representational versus dispositional memories because of its simple operational designation. *Dispositional* memory is implied if correct discriminations are made when the necessary stimuli are available to the sensorium at the time of choice; *representational* memory, similar to recognition-recall in humans, requires (in addition) information not present in the sensorium but represented in the brain. Experimentally, a non-matching to sample task is employed with rats in a T-maze, recording percentage of correct choices and run times. Lesion [10] and pharmacologic [97] studies using this paradigm have demonstrated the importance of the septo-hippocampal pathway and muscarinic cholinergic system for representational memory.

Antimuscarinic-induced deficit and subsequent adaptation. Recently, in this laboratory it has been shown that the M_1-selective antagonist pirenzepine produces a representational memory deficit subsequent to its injection directly into the hippocampi of trained rats [98]. On the other hand, the less selective antagonist scopolamine produced increases in response times as well as decreases in percentage of correct responses associated with a specific representational memory deficit. The data suggest that M_1 receptors in the hippocampus may be involved specifically in representational memory processing. The finding that gallamine, which has a low affinity for hippocampal structures, was unable to antagonize excitatory responses in CA1 pyramidal cells elicited by muscarinic agonists [99] is consistent with this view. Behavioral adaptation, which develops for both muscarinic antagonists (and which may be an expression of drug tolerance), is seen as an increased ability to perform the task with repeated exposure to the drugs [98].

Receptor upregulation. To examine the possible role of muscarinic receptors in the development of adaptation to the antagonists, muscarinic receptors were examined autoradiographically after the conclusion of behavioral testing [100]. Animals that developed tolerance (i.e. adapted) to pirenzepine displayed an increase in receptor number in all areas of the cortex and in the CA3–CA4 region of the hippocampus as well. This receptor upregulation was not seen in animals that were tolerant to scopolamine.

Animals that were tolerant to the two antagonists could be differentiated further on the basis of affinities of muscarinic ligands measured indirectly by their abilities to displace [^{3}H]QNB in coronal sections *in vitro*. The affinities for both antagonists, but not carbachol, were decreased in the same areas showing receptor upregulation for the animals tolerant to pirenzepine. Receptors from rats that were adapted to scopolamine displayed increases in affinity for carbachol in other brain areas. These results suggest that the increased receptor number together with decreased affinity for antagonists may be involved in the mediation of behavioral tolerance to pirenzepine, while increases in agonist affinity are associated with adaptation to scopolamine. The results lend support to the hypothesis that muscarinic receptor subtypes reflect functional differences that can be selectively examined *in vivo*.

Perspectives for future research

Taken together, the binding data indicate the existence of multiple muscarinic receptors in the CNS. Receptors in the brainstem appear fundamentally different from those in the forebrain in terms of ligand specificity and regulation by metals, sulfhydryl reagents, and GTP. A role for protein sulfhydryl groups in regulating forebrain receptors is strongly implied by the effects of sulfhydryl alkylating reagents and metals *in vitro* and the effects of Cu^{2+} deficiency *in vivo*.

Comparing ligand specificities for various biochemical responses in different tissues and clonal cell lines suggests the lack of a simple correspondence between receptor subtypes as defined by the binding of selective ligands and activation of specific second messenger systems. This underscores the need to demonstrate muscarinic receptor-mediated processes directly in various brain areas (in addition to peripheral systems and cultured cells).

Although there remains some controversy regarding the nature of the binding of gallamine, the available evidence indicates that pirenzepine and gallamine are selective antagonists for forebrain (M_1) and brainstem (M_2) receptors respectively. Fore-

brain muscarinic receptors are more sensitive to pirenzepine than gallamine by a number of criteria, including selectivity for binding to the receptors and inhibition of carbachol-stimulated PI turnover and GTPase activity. In addition, pirenzepine is a potent and specific drug for impairing performance on a task that measures representational memory in animals. It will be important to compare the two antagonists for blocking of the muscarinic agonist-induced inhibition of adenylate cyclase and inhibition of PI turnover and GTPase in areas such as brainstem where gallamine has a higher affinity for muscarinic receptors.

It will also be important to elucidate the possible role of a GTP-binding protein in coupling muscarinic receptors to PI turnover in the forebrain. Stimulation of PI turnover has until very recently required whole cells, and it is difficult to manipulate intracellular levels of GTP. New methods that permit the measurement of PI turnover in broken cell preparations are currently emerging, providing perhaps the necessary tools for a direct demonstration of a GTP effect on this response [101]. As the pharmacology of the PI turnover cycle emerges, it may be possible to augment receptor-directed approaches with agents that affect the second messenger system directly, especially if there is some specificity with respect to the components of the muscarinic receptor-linked PI turnover system.

Several independent lines of evidence indicate the plasticity of central muscarinic receptors *in vivo*. The occurrence of a reversible alteration in the affinity of forebrain muscarinic receptors for agonists (in Cu^{2+}-deficient rats) and the upregulation of muscarinic receptors after multiple exposures to pirenzepine, which may be related to the behavioral adaptation to the drug, are two examples. Determination of receptor subtype distribution and behavioral testing of rats that are minimally Cu^{2+}-deficient may elucidate further the putative role of sulfhydryl groups in regulating M_1 receptors and their possible role in memory tasks. It will also be important to address whether the apparent increases in receptors after multiple exposures to pirenzepine are the result of increased receptor synthesis or some other factor(s).

The regional selectivity of several muscarinic agonists and antagonists in the brain is striking. Receptor autoradiography provides a convenient tool for screening any muscarinic ligand for its regional selectivity. Promising candidates can be tested at the biochemical level by measuring their effects on second messenger systems. Muscarinic agents that prove selective at the receptor binding and biochemical levels can be tested in behavioral paradigms that measure representational memory in animals. It is expected that this three-tiered approach—screening of muscarinic ligands for regional selectivity by autoradiography, measurement of specificities of regionally selective ligands for local second messenger systems, and behavioral testing of specific ligands—will lead most efficiently to new therapeutic strategies for cognitive and other deficits involving central cholinergic neurons.

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REFERENCES

1. G L. Peterson, G. S. Herron, M. Yamaki, D. S. Fullerton and M. I. Schimerlik, *Proc. natn. Acad. Sci. U.S.A.* **81**, 4993 (1984).
2. K. Haga and T. Haga, *J. biol. Chem.* **260**, 7927 (1985).
3. L. G. Abood and J. H. Biel, *Int. Rev. Neurobiol.* **4**, 218 (1962).
4. L. G. Abood, in *Drugs Affecting the Central Nervous System* (Ed. A. Burger), p. 127. Marcel Dekker, New York (1968).
5. P. Davies and A. J. F. Maloney, *Lancet* **2**, 1403 (1976).
6. E. K. Perry, R. H. Perry, G. Blesser and B. E. Tomlison, *J. neurol Sci.* **34**, 247 (1977).
7. R. T. Bartus, R. L. Dean, B. Beer and A. S. Lippa, *Science* **217**, 408 (1982).
8. P. J. Whitehouse, D. L. Price, R. G. Struble, A. W. Clark, J. T. Coyle and M. R. De Long, *Science* **215**, 1237 (1982).
9. M. Weiner, *Geriatrics* **37**, 81 (1982).
10. G. J. Thomas and P. S. Spafford, *Behavl Neurosci.* **98**, 394 (1984).
11. H. E. Brezenoff, *Fedn Proc.* **43**, 17 (1984).
12. J. H. Ashe and C. A. Yarosh, *Neuropharmacology* **23**, 1321 (1984).
13. S. K. Fisher and R. T. Bartus, *J. Neurochem.* **45**, 1085 (1985).
14. R. A. Gonzales and F. T. Crews, *J. Neurochem.* **45**, 1076 (1985).
15. R. Hammer, C. P. Berrie, N. J. M. Birdsall, A. S. V. Burgen and E. C. Hulme, *Nature, Lond.* **283**, 90 (1980).
16. J. Ellis and W. Hoss, *Biochem. Pharmac.* **31**, 873 (1982).
17. R. S. Aronstam, W. Hoss and L. G. Abood, *Eur. J. Pharmac.* **46**, 279 (1977).
18. R. S. Aronstam, L. G. Abood and W. Hoss, *Molec. Pharmac.* **14**, 575 (1978).
19. J. R. Farrar and W. Hoss, *Biochem. Pharmac.* **33**, 2849 (1984).
20. C. P. Berrie, N. J. M. Birdsall, A. S. V. Burgen and E. C. Hulme, *Biochem. biophys. Res. Commun.* **87**, 1000 (1979).
21. F. J. Ehlert, W. R. Roeske, L. B. Rosenberger and H. I. Yamamura, *Life Sci.* **26**, 245 (1980).
22. M. Sokolovsky, D. Gurwitz and R. Galron, *Biochem. biophys. Res. Commun.* **94**, 487 (1980).
23. D. Gurwitz and M. Sokolovsky, *Biochem. biophys. Res. Commun.* **96**, 1296 (1980).
24. J. R. Farrar, W. Hoss, R. M. Herndon and M. Kuzmiak, *J. Neurosci.* **5**, 1083 (1985).
25. N. J. M. Birdsall, E. C. Hulme and A. S. V. Burgen, *Proc. R. Soc. Biol.* **207**, 1 (1980).
26. M. Watson, H. I. Yamamura and W. R. Roeske, *Life Sci.* **32**, 3001 (1983).
27. W. Hoss, M. Price and W. S. Messer, Jr., *Trends pharmac. Sci.* (Suppl.), 94 (1986).
28. J. K. Wamsley, M. A. Zarbin, N. J. M. Birdsall and M. J. Kuhar, *Brain Res.* **200**, 1 (1980).
29. J. K. Wamsley, D. R. Gehlert, W. R. Roeske and H. I. Yamamura, *Life Sci.* **34**, 1395 (1984).
30. D. Gurwitz, Y. Kloog and M. Sokolovsky, *Proc. natn. Acad. Sci. U.S.A.* **81**, 3650 (1984).
31. F. J. Ehlert, Y. Dumont, W. R. Roeske and H. I. Yamamura, *Life Sci.* **26**, 961 (1980).
32. N. J. M. Birdsall, A. S. V. Burgen and E. C. Hulme, *Molec. Pharmac.* **14**, 723 (1978).

33. E. Horvath, J. Traber and D. G. Spencer, Jr., *Trends pharmac. Sci.* (Suppl.), 93 (1986).
34. W. S. Messer Jr. and W. Hoss, *Soc. Neurosci. Abstr.* **10**, 381 (1984).
35. M. Watson, W. R. Roeske and H. I. Yamamura, *Life Sci.* **31**, 2019 (1982).
36. J. M. Stockton, N. J. M. Birdsall, A. S. V. Burgen and E. C. Hulme, *Molec. Pharmac.* **23**, 551 (1983).
37. J. Ellis and R. H. Lenox, *Biochem. Pharmac.* **34**, 2214 (1985).
38. D. C. Mash, D. D. Flynn and L. T. Potter, *Science* **228**, 115 (1985).
39. M. McKinney and J. T. Coyle, *J. Neurosci.* **2**, 97 (1982).
40. J. Ellis and R. H. Lenox, *Biochem. biophys. Res. Commun.* **126**, 1242 (1985).
41. J-H. Lee and E. El Fakahany, *J. Pharmac. exp. Ther.* **233**, 707 (1985).
42. M. C. Olianas, P. Onali, N. H. Neff and E. Costa, *Molec. Pharmac.* **23**, 393 (1983).
43. R. B. Meeker and T. K. Harden, *Molec. Pharmac.* **22**, 310 (1982).
44. M. R. Hanley and L. L. Iversen, *Molec. Pharmac.* **14**, 246 (1978).
45. J. A. Ferrendelli, A. L. Steiner, J. R. McDougal and D. M. Kipnis, *Biochem. biophys. Res. Commun.* **41**, 1061 (1970).
46. J. A. Ferrendelli, E. H. Rubin and D. A. Kincerf, *J. Neurochem.* **26**, 741 (1976).
47. N. M. Nathanson, W. L. Klein and M. Nirenberg, *Proc. natn. Acad. Sci. U.S.A.* **75**, 1788 (1978).
48. S. B. Masters, T. K. Harden and J. H. Brown, *Molec. Pharmac.* **26**, 149 (1984).
49. S. B. Masters, M. T. Quinn and J. H. Brown, *Molec. Pharmac.* **27**, 325 (1985).
50. L. Stryer, J. B. Hurley and B. K. K. Fung, *Trends biochem. Sci.* **6**, 245 (1981).
51. K. H. Jakobs, K. Aktories and G. Schultz, *Naunyn-Schmiedeberg's Archs Pharmac.* **310**, 113 (1979).
52. K. H. Jakobs, K. Aktories and G. Schultz, in *Advances in Cyclic Nucleotide and Protein Phosphorylation Research* (Eds. P. Greengard, G. A. Robison, R. Paoletti and S. Nicosia), Vol. 17, p. 135. Raven Press, New York (1984).
53. T. Murayama and M. Ui, *J. biol. Chem.* **258**, 3319 (1983).
54. H. Kurose, T. Katada, T. Amano and M. Ui, *J. biol. Chem.* **258**, 4870 (1983).
55. D. Cassel and T. Pfeuffer, *Proc. natn. Acad. Sci. U.S.A.* **75**, 2669 (1978).
56. M. E. Abood, J. B. Hurley, M-C. Pappone, H. R. Bourne and L. Stryer, *J. biol. Chem.* **257**, 10540 (1982).
57. C. Van Dop, G. Yamanaka, F. Steinberg, R. Sekura, C. R. Manclark, L. Stryer and H. R. Bourne, *J. biol. Chem.* **259**, 23 (1984).
58. A. R. Hughes, M. W. Martin and T. K. Harden, *Proc. natn. Acad. Sci. U.S.A.* **81**, 5680 (1984).
59. T. Evans, M. W. Martin, A. R. Hughes and T. K. Harden, *Molec. Pharmac.* **27**, 32 (1985).
60. A. M. Pappenheimer, Jr., *A. Rev. Biochem.* **46**, 69 (1977).
61. D. Cassel and Z. Selinger, *Biochim. biophys. Acta* **452**, 538 (1976).
62. P. Onali, M. C. Olianas, J. P. Schwartz and E. Costa, *Molec. Pharmac.* **24**, 380 (1983).
63. D. Cassel and Z. Selinger, *Proc. natn. Acad. Sci. U.S.A.* **75**, 4155 (1978).
64. P. H. Franklin and W. Hoss, *Trends pharmac. Sci.* (Suppl.), 82 (1986).
65. D. W. Gil and B. B. Wolfe, *J. Pharmac. exp. Ther.* **232**, 608 (1985).
66. S. K. Fisher, P. D. Klinger and B. W. Agranoff, *J. biol. Chem.* **258**, 7358 (1983).
67. R. H. Michell, *Biochim. biophys. Acta* **415**, 81 (1975).
68. C. P. Downes, *Cell Calcium* **3**, 413 (1982).
69. M. J. Berridge, C. P. Downes and M. R. Hanley, *Biochem. J.* **206**, 587 (1982).
70. F. J. Monsma and W. Hoss, *Soc. Neurosci. Abstr.* 96 (1985).
71. X. Tien and L. J. Wallace, *Biochem. Pharmac.* **34**, 588 (1985).
72. J. H. Brown and D. Goldstein, *Trends pharmac. Sci.* (Suppl.), 83 (1986).
73. M. J. Berridge, *Biochem. J.* **220**, 345 (1984).
74. J. M. Baraban, S. H. Snyder and B. E. Alger, *Proc. natn. Acad. Sci. U.S.A.* **82**, 2538 (1985).
75. M. Castagna, Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa and Y. Nishizuka, *J. biol. Chem.* **257**, 7847 (1982).
76. J. Jolles, H. Zwiers, C. J. van Dongen, P. Schotman, K. W. A. Wirtz and W. H. Gispen, *Nature, Lond.* **286**, 623 (1980).
77. C. A. Sutton and T. F. J. Martin, *Endocrinology* **110**, 1273 (1982).
78. P. M. Hinkle and W. J. Phillips, *Proc. natn Acad. Sci. U.S.A.* **81**, 6183 (1984).
79. R. S. Aronstam, D. C. Schnessler and M. E. Eldefrawi, *Life Sci.* **23**, 1377 (1978).
80. S. Carson, *Biochem. Pharmac.* **31**, 1806 (1982).
81. M. Gavish and M. Sokolovsky, *Biochem. biophys. Res. Commun.* **109**, 434 (1982).
82. J. Baumgold, *Soc. Neurosci. Abstr.* **11**, 655 (1985).
83. G. S. Herron, S. Miller, W-L. Manley and M. I. Schimerlick, *Biochemistry* **21**, 515 (1982).
84. B. Baron and L. G. Abood, *Life Sci.* **35**, 2407 (1984).
85. V. A. Florio and P. C. Sternweis, *J. biol. Chem.* **260**, 3477 (1985).
86. S. Avissar, G. Amitai and M. Sokolovsky, *Proc. natn. Acad. Sci. U.S.A.* **80**, 156 (1983).
87. S. K. Smith and L. E. Limbird, *Proc. natn. Acad. Sci. U.S.A.* **78**, 4026 (1981).
88. B. F. Kilpatrick and M. G. Caron, *J. biol. Chem.* **258**, 13528 (1983).
89. J. Ellis and W. Hoss, *Brain Res.* **193**, 189 (1980).
90. D. A. Drachman and J. L. Leavitt, *Archs Neurol., Chicago* **30**, 113 (1974).
91. B. T. Hyman, G. W. Van Hoesen, A. R. Damasio and C. L. Barnes, *Science* **225**, 1168 (1984).
92. M. J. Ball, *Acta neuropath.* **37**, 111 (1977).
93. O. Nordstrom, P. Alberts, A. Westlind, A. Unden and T. Bartfai, *Molec. Pharmac.* **24**, 1 (1983).
94. W. James, *The Principles of Psychology*, Vol. 1. Henry Holt, New York (1890).
95. W. K. Honig, in *Cognitive Processes in Animal Behavior* (Eds. S. H. Hulse, H. Fowler and W. K. Honig), p. 211. Erlbaum, Hillsdale, NJ (1978).
96. M. E. Stanton, G. J. Thomas and G. N. O. Brito, *Behavl Neurosci.* **98**, 405 (1984).
97. J. W. Kasckow, G. J. Thomas and R. M. Herndon, *Physiol. Psychol.* **12**, 111 (1984).
98. W. S. Messer, Jr., W. Hoss and G. J. Thomas, *Soc. Neurosci. Abstr.* **11**, 1294 (1985).
99. A. E. Cole and R. A. Nicoll, *Brain Res.* **305**, 283 (1984).
100. W. S. Messer, Jr., W. Hoss, G. J. Thomas and M. Price, *Trends pharmac. Sci.* (Suppl.), 95 (1986).
101. S. Cockcroft and B. D. Gomperts, *Nature, Lond.* **314**, 534 (1985).