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THE COUPLING OF THE NEURONAL MUSCARINIC RECEPTOR TO RESPONSES

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MUSCARINIC RECEPTORS

Introduction

Muscarinic acetylcholine receptors are plasma membrane proteins that bind the neurotransmitter acetylcholine and by their conformational changes elicit specific biological events within cells. They are pharmacologically defined by their binding selectivity for a specific class of drugs, among which are the agonists muscarine and oxotremorine and the various atropine-like antagonists. Muscarinic receptors mediate various types of responses in cardiac muscle, in numerous smooth muscles, in exocrine glands, throughout the peripheral and central nervous system, and in some kinds of cultured nerve cells. Usually two or three subtypes of muscarinic receptors exist, distinguished by their relative binding affinity for agonists (1). Though the types of biological responses among these tissues are varied, the respective proteins that bind the agonist appear to be similar or identical in binding properties and molecular size. It is probably their coupling to unique tissue-specific effectors that gives rise to varied responses.

Responses mediated by muscarinic receptors are slow in onset and development and thus are distinctly different from nicotinic responses, but like nicotinic responses they can desensitize. In the brain, where the muscarinic receptor is the predominant cholinergic receptor, its activation can lead to either excitation or inhibition of neurons (2). In the cerebral cortex, for example, pyramidal cells become depolarized from decreases in potassium (K^+) conductance (3),

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while in the ventral thalamus inhibitory responses are encountered (4). In cardiac muscle, hyperpolarization results from increases in K^+ conductance (2). In a tissue where there are no action potentials, the parotid gland, muscarinic receptors trigger calcium (Ca^{+2}) influx from a neurotransmitter-sensitive pool, probably located in plasma membrane binding sites, that then mediates secretion of amylase (5). An apparently separate but Ca^{+2}-dependent response in this gland is K^+ efflux (6). Muscarinic receptors in smooth muscle can cause either contraction or relaxation or can modulate spontaneously contracting muscle (7). K^+ efflux in smooth muscle may also be a separate response. In many tissues with muscarinic receptors there are often additional responses of a biochemical nature. The stimulation of guanylate cyclase (8–14) and/or the inhibition of adenylate cyclase (12, 13, 15–21) is often observed; these responses are often dependent upon the presence of Ca^{+2} in the extracellular medium. The metabolism of phospholipids is sometimes a consequence of muscarinic activation (22–24) and there are some recent data implicating arachidonic acid metabolism (25, 26). Most of these biochemical responses have been observed to desensitize (27–29).

Many of the responses cited conveniently occur in "neuron-like" murine neuroblastoma cells (for example, clone N1E-115), and thus these cells have been extensively employed as model systems to study the function of the muscarinic receptor. Studies of the muscarinic receptors of neuroblastoma provide valuable supplementary information to studies of the receptor in the nervous system. Neuroblastoma systems are particularly useful for asking questions at the molecular level, where such approaches are very difficult with in vivo systems.

Memory is one example of a brain function in which more knowledge of muscarinic processes at the molecular level would be useful. According to current thought, memory involves cholinergic systems innervating the cerebral cortex and hippocampus (30). Muscarinic receptors are probably involved in mediating this input. Antimuscarinic agents, apparently by blocking these receptors, can cause memory processing impairments similar to those seen in the aged (31). A possible pathologic correlate of pharmacologic blockade of cholinergic input to these muscarinic receptors is Alzheimer's disease, where a profound lesion of the basal forebrain source of cholinergic innervation of the cerebral cortex and hippocampus apparently leads to deficits in cognitive function (32). As this disease progresses, the patient loses his ability to process recent memory. In the later stages deterioration in general cognitive function occurs; in the terminal phase there is profound dementia. The exact changes that occur in the muscarinic receptor system in response to the absence of cholinergic input and to subsequent administration of drugs in substitution or precursor therapy are not known. There is some information from binding studies with homogenates of human brain indicating up-regulation (33), but the

details of the biochemical response of the receptor to denervation are not known. Conceivably, such basic knowledge, much of which is coming from studies of receptors in cultured cells, could contribute to successful drug therapy for Alzheimer's dementia and perhaps other brain disorders that have a component of dementia or memory dysfunction. The advantages of studying the muscarinic receptor of intact N1E-115 cells have been exemplified by the finding that many psychotherapeutic agents, including antidepressants and neuroleptics (34) and local anesthetics (35), mediate effects at these receptors [blockade of guanosine cyclic monophospate (cGMP) formation (36)].

The Biochemical Nature of the Receptor

The muscarinic receptor can be solubilized from membranes and studied in extracts with high-affinity reversible ligands or with irreversible alkylating ligands, but because of its instability and low abundance the receptor has not been purified. Several proteins with muscarinic binding sites were detached from membranes of rat brain with high salt concentrations; the putative receptor had a molecular weight of only 30,000 daltons (37). More recently, the receptor was affinity labeled, solubilized with detergents, and studied by SDS gel electrophoresis (38, 39). The receptor from rat brain and smooth muscle had an estimated molecular weight of 80,000 daltons and migrated as a single band (38). Affinity labeling was largely prevented by atropine, which indicates the receptor's muscarinic nature. The affinity-labeled receptor of mouse cerebral cortex, where the low-affinity receptor is prevalent, had a molecular weight of 86,000 daltons (39). Affinity labeling of the muscarinic receptor in the mouse brain stem, where there are mostly high-affinity receptors, revealed both 86,000 and 180,000 dalton proteins. Mild alkaline hydrolysis converted these proteins into 40,000 dalton fragments. It was suggested that the low-affinity receptor was a dimer and the high-affinity receptor was a tetramer of 40,000-dalton, covalently coupled subunits. This was supported by the apparent interconversion of sites by a guanosine triphosphate (GTP) analog or by manganese (Mn^{+2}), which are known to have such effects on binding to mouse brain muscarinic receptors (40, 41).

The brain receptor has been shown by lesion experiments to be located for the most part post-synaptically. A small fraction of the binding sites are tentatively identified as pre-synaptic in cortex (42) and hippocampus (43), where there is also physiological evidence for their presence (44). After subcellular fractionation of the brain tissue, the muscarinic receptor is found in the synaptosomes (45). In autoradiographic experiments, receptors can be visualized over cell bodies or dendritic fields of neurons (46). Thus, binding studies of the brain can be expected to provide information about synaptically located receptors. In general, antagonists bind to a single class of sites with rank order of affinities that correlate very well with their potencies in the

blockade of cholinergic function in intact tissues (47). However, occupancy curves for agonists are multiphasic, with Hill slopes less than unity, and their Scatchard transforms are concave upward (48–50). This phenomenon is due to the presence of multiple independent binding sites for agonists, which sometimes can apparently be induced to interconvert in vitro. It was shown in rat cortex that the low Hill slopes for agonists were not due to negative cooperativity by using an affinity alkylating agent to partially occlude sites before performing binding with carbachol (48). Though there has been some evidence indicating cooperativity in the binding of the receptor in the pituitary (51), the brain itself has generally been found to possess independent agonist sites (48, 50). Studies with iterative computer methods for analyzing binding data have shown that two agonist sites (high- and low-affinity) predominate in brain and heart, and that another one of higher affinity ("super-high") is also present in low concentrations in the brain (52). In the brain, the proportions of these sites vary with region but a substantial number of muscarinic agonists bind to these three sites in a given brain region in virtually the same ratios of capacities (52). Agonists do not seem to induce multiple-site phenomena in vitro; thus, it would be expected that these more-or-less fixed-capacity multiple binding sites in some way reflect functional heterogeneity. N1E-115 cells possess low- and high-affinity agonist sites (53).

Since a single receptor protein of 86,000 daltons (or a multiple of it) is able to produce multiphasic binding, the most parsimonious explanation for this apparent heterogeneity is that it is attributable to differences in the receptor's conformation, perhaps to its chemical modification, or to its coupling with another protein (54). The affinity-labeling data cited above, for example, suggest that heterogeneity results from the covalent intercoupling of subunits. Guanyl nucleotides (GTP, etc) have an effect on agonist binding to the muscarinic receptor that is similar to that seen with the β-adrenergic receptor: agonist occupancy curves are shifted to the right (40, 41, 55, 56). This GTP effect is variable with brain region: the brain-stem receptors show a substantial shift similar to that observed in the heart (55), but cortical receptors display little or no change in the dose with which the agonist inhibits [^{3}H]-antagonist binding by 50% (IC_{50}).

Computer analyses of these effects have indicated that heart or brain-stem high-affinity sites are possibly being converted into low-affinity sites (40, 54). By analogy with the β-adrenergic receptor, this suggests that the low-affinity site, at least in the brain-stem, is coupled to responses. It has been shown that incubating synaptosomes under phosphorylating conditions partially inhibits this rightward shift, though the receptor itself is not phosphorylated (57). Other agents, such as certain metal ions, apparently have been able to change the proportions of sites seen in broken-cell preparations (41, 54). Although these observations are suggestive of regulatory mechanisms in vivo, the physiological meaning for multiple agonist binding sites in the brain remains unclear. It is

possible that either receptors are coupled to different effectors for unique responses or that multiple sites are features of a regulatory mechanism involving a single effector. It is also possible that heterogeneity is an artifact of homogenization. The effects of GTP on binding to the brain-stem receptor is evidence in favor of genuine functional differences between these sites.

When the neuronal muscarinic receptor is solubilized with detergents, its agonist-binding heterogeneity disappears (58). The solubilized receptor-detergent complex binds carbachol with a binding affinity very much like that of the low-affinity agonist site in the membrane. If the coupling is disrupted by digitonin solubilization, this would mean that the high-affinity form is the coupled receptor. But this presumes that the conformations of the membrane-bound and solubilized receptor are the same. A body of physiological evidence suggests that the low-affinity membrane-bound receptor of a variety of tissues is coupled to responses:

1. Birdsall et al have found that the equilibrium binding constants for agonists at the brain low-affinity agonist site (K_L) correlate roughly with the potencies of these agonists for contraction of smooth muscle when spare receptors are accounted for (48).
2. The negative chronotropic response of chick atria in vitro correlates with the occupancy of the low-affinity receptor of this tissue (59).
3. The dose of carbachol effective for 50% down-regulation of the receptor of intact heart cells in culture is at a value typical of K_L in brain or neuroblastoma (60).
4. The half-maximal dose for carbachol's stimulation of K^+ efflux of these heart cells and its desensitization is in the range of the neuronal K_L (60).
5. In N1E-115 cells the dose for half-maximal cGMP response (ED_{50}) for several agonists correlates almost exactly with the K_L for these agonists and not their K_H (equilibrium dissociation constant for the high-affinity site) (61). However, the N1E-115 muscarinic inhibition of prostaglandin E_1–stimulated adenosine cyclic monophosphate (cAMP) formation correlates with K_H (62).
6. In synaptosomal preparations of cerebral cortex the dose-response for muscarinic mediation of phosphatidylinositol turnover correlates with K_L (63). With N1E-115 the turnover of phosphatidylinositol has an ED_{50} for carbachol at 10μM, a concentration in at least tenfold excess of K_H (28).
7. In slices of the rat striatum, Hanley & Iversen showed that for the formation of cGMP by various muscarinic agonists the ED_{50}'s were near their K_L's (9).

Thus, there is considerable evidence that the low-affinity agonist site is a "functional" site in vivo. However, as has been noted (1), in a given tissue the ED_{50}'s for stimulation of various responses can differ widely. Thus, there may well be responses coupled to the high-affinity or super-high-affinity

receptors. A good example of this is the muscarinic receptor of pancreatic acini, where amylase secretion seems to involve high-affinity receptors (64). It is not yet known if the agonist binding sites are located on the same or different neurons in brain tissue, but high- and low-affinity sites do appear to be differentially distributed in cortical cell layers (65). N1E-115 cells possess both predominant agonist sites in the same proportion as those in whole rat cerebral cortex (53).

The binding of antagonists by the muscarinic receptor has sometimes appeared multiphasic. Pirenzepine has been shown to bind to multiple sites in the brain as well as in other tissues (66); it labels two sites in human cerebral cortex in the same approximate proportions as the rat cortical high- and low-affinity receptors (67). In synaptosomes of the cortex of cat brain, [^{3}H]-quinuclidinyl benzilate (^{3}H-QNB) was observed to associate to two sites in roughly the same proportion as the two predominant agonist sites (68). In the adenohypophysis there seem to be two antagonist sites (69). In frog heart, two antagonist sites for ^{3}H-QNB are thought to correlate with two agonist sites for oxotremorine, and interconversion of these sites appears to be effected by guanine nucleotides (70). The meaning of multiple antagonist sites remains elusive and somewhat controversial. One possible explanation for binding heterogeneity with antagonists is that, under the appropriate conditions, it reflects the coupling phenomenon, as agonists appear to. It is not a general finding in brain tissue. There has been one report of two antagonist sites in N1E-115 and NG108-15 neuroblastoma cells (71).

The brain muscarinic receptor apparently has a sulfhydryl group that is important for its conformation (72, 73). Alkylation of receptors in membranes with N-ethylmaleimide (NEM), which is selective for sulfhydryl groups, seems to convert the agonist binding sites into a higher affinity state (74, 75). However, the opposite effect with NEM has also been observed in brain (76). The inconsistency of NEM effects as observed in brain tissue requires a conservative view of the value of NEM in site conversion. The degree of chemical modification of the sulfhydryls of the brain receptor can be varied by the presence of ligands at the binding site and, with agonists at least, this is due to an induced conformational change (77). Reducing agents like dithiothreitol can decrease the binding of an antagonist or agonist to the brain receptor (72, 75); this is suggestive of the importance of a disulfide bond in the conformation of the protein.

Most metal ions do not affect binding except at high concentrations (78). Ca^{+2} ions at physiological concentrations do not change binding, but the lanthanides, which are antagonists at Ca^{+2}-binding sites, affect muscarinic binding (78, 79) and also affect mediation of cGMP formation in N1E-115 cells. Transitional metal ions affect receptor binding in rat (78) and mouse (41)

cerebral cortex and cGMP formation in N1E-115 cells (80, 81). Mn^{+2}, nickel ions (Ni^{+2}), and cobalt ions (Co^{+2}) seem to convert mouse low-affinity sites to high-affinity sites, which is interesting in light of the ability of Mn^{+2} and Ni^{+2} to stimulate cGMP formation in N1E-115 cells (80, 81). The effects of these ions could be due to their action at receptor sulfhydryl groups, which seem to be important for the receptor's conformation, or at Ca^{+2} binding sites on the receptor or on neighboring phospholipids. Ca^{+2} has been shown to bind to acidic phospholipids (82) and this is affected by phenytoin, which is a blocker of Ca^{+2}-dependent cGMP formation in N1E-115 cells (83). It has been found that very high monovalent metal ion concentrations will obscure differences in binding affinities of the high- and low-affinity sites in cortex (54); it may be then that sites within a given region are identical polypeptides with various conformations as dictated by their ionic environment. However, in a study of the effects of metals on antagonist binding to the rat brain receptor, Ikeda et al found that the effect of metal ions at high concentrations was different, even opposite, for forebrain and brainstem areas (74). These workers suggested that the concept of a single receptor protein with two conformational states of differing agonist binding affinities, with relative proportions of the states being a function of brain region, could not fully explain this particular effect of metals (74).

The phospholipids of the membrane may act on the receptor to affect its conformation. Exogenous phosphatidylserine, phospatidic acid, and phosphatidylinositol enhance ^{3}H-QNB binding to the receptor in homogenates (84). The types of fatty acyl groups in the phospholipids are probably also important, since exogenous unsaturated but not saturated fatty acids, or treatment of the membranes with phospholipase A, will inhibit ^{3}H-QNB binding (84). The effect of changing the lipid environment of the receptor on agonist binding has not been investigated, but it is possible that lipids are involved in the mechanism of agonist-induced regulation of the receptor. The type and concentration of unsaturated lipids present in the membrane phospholipids are known to be important in plasma membrane processes; for example, the growth of PC12 pheochromocytoma cells with various unsaturated fatty acids in the media changes the composition of the membrane phospholipid acyl groups and profoundly affects opiate receptor binding (85); exogenous arachidonic acid reduces γ-aminobutyric acid and glutamate uptake and Na^+/K^+-ATPase activity in brain slices and synaptosomes (86). Membrane fluidity, a function of lipid composition, is important in muscarinic processes, as can be seen by the effect of temperature on carbachol-stimulated cGMP formation in N1E-115 cells: receptor-mediated cyclic GMP formation and the desensitization and resensitization of the effect will not occur below 21°C, which is in the vicinity of the transition temperature for lipid bilayers (87).

MOLECULAR CONSEQUENCES OF RECEPTOR ACTIVATION

Changes in Electrical Properties of the Cell

Slow depolarization of the plasma membrane is commonly observed in the central nervous system (CNS) in response to iontophoresis of muscarinic agonists (2). This raises the level of excitability of the neuron and slowly increases spontaneous firing. This is in contrast to the rapid depolarization and increased firing rate elicited by an agent like glutamate.

Particularly well-studied is the muscarinic response of the pyramidal cells of cerebral cortex, where there is a typical slowing of firing for about 5–10 seconds, followed by a long period of increased firing rate caused by gradual depolarization (3). These excitatory effects of acetylcholine have been shown to be mediated by a decrease in the resting membrane conductance for K^+ and by a partial inactivation of voltage-sensitive K^+ channels that repolarize the cell (3). The action of acetylcholine on cortical neurons is blocked by the metabolic inhibitor dinitrophenol (DNP), which does not inhibit the stimulation of these cells by glutamate except in high doses (88). This indicates that the post-synaptic muscarinic action in vivo involves metabolic processes. The ionic basis for inhibitory responses, such as those found in the thalamus, is not known.

There is some evidence that Ca^{+2} is necessary for muscarinic effects on K^+ conductance in the CNS. Barium ion (Ba^{+2}), which is Ca^{+2}-like, excites cortical neurons in a way similar to acetylcholine, by reduction in K^+ conductance, and these neurons also usually respond to acetylcholine (89). It is possible that Ba^{+2} substitutes for Ca^{+2} in some Ca^{+2}-dependent process; Ba^{+2} and Sr^{+2} can substitute for Ca^{+2} in the muscarinic stimulation of cGMP formation in rat ductus deferens (90) and Ba^{+2} can pass through Ca^{+2} channels (91). A role for internal Ca^{+2} in modulation of K^+ conductance has been shown in spinal motor neurons, where intracellular injection of Ca^{+2} increases the K^+ conductance (92) and injection of EGTA blocks K^+ conductance (93).

Differentiated N1E-115 cells can generate action potentials and the voltage-dependent sodium ion (Na^+), K^+, and Ca^{+2} channels of these cells have been shown to be very similar to other well-characterized neuronal channels (94). The voltage-sensitive Ca^{+2} channel is relatively weak and is blocked by Co^{+2}, Mn^{+2}, or the lanthanides (95). Activation of this channel by increasing the external K^+ concentration to a depolarizing level can cause cGMP formation in N1E-115 cells (96). N1E-115 cells have another K^+ channel that is triethylamine(TEA)-resistant and Ca^{+2}-dependent, which can give rise to a prolonged after-hyperpolarization (AHP) (97). Activation of muscarinic receptors of N1E-115 cells, in addition to causing cGMP formation, also causes the cells to become hyperpolarized (98). Neurotransmitter-mediated cGMP formation,

which occurs in both differentiated and undifferentiated cells, is a Ca^{+2}-dependent process. However, it is not due to activation of voltage-sensitive Ca^{+2} channels. Carbachol's stimulation of cGMP formation is additive to cGMP formation mediated by high K^+ depolarization (96). Thus, to mediate the hyperpolarization the neurotransmitter receptor and the action potential may be indirectly operating the same TEA-insensitive Ca^{+2}-dependent K^+ channels, both by separate Ca^{+2}-mediated pathways. This idea of separate Ca^{+2} channels is supported by the finding that the dihydropyridines inhibit cGMP formation mediated via the voltage-sensitive Ca^{+2} channel but do not inhibit the neurotransmitter-stimulated cGMP formation (R. M. Snider, E. Richelson, unpublished information). It is thus conceivable that there are additional steps between Ca^{+2} influx and the effect on these delayed K^+ channels in N1E-115 cells. These steps may be the stimulation of guanylate cyclase and some cGMP-dependent action on K^+ channels, perhaps the phosphorylation of the K^+ channel by a cGMP-dependent protein kinase.

A role for cGMP in membrane events in the muscarinic activation of cortical neurons has been implicated by recent experiments. When cyclic GMP is iontophoresed on to cortical pyramidal cells an increase in their firing rate follows (99). Intracellular injection of cGMP into cortical pyramids causes an increase in membrane resistence and increased firing identical to that caused by acetylcholine when it is iontophoresed on to the same cell extracellularly (100). In these carefully controlled experiments, blockade of acetylcholine effects but not cGMP effects by atropine showed that the receptor involved was muscarinic. To draw a parallel, then, perhaps Ca^{+2}-dependent K^+ channels in both the brain and in N1E-115 cells mediate the muscarinic electrical responses, either excitatory or inhibitory, contingent upon or in parallel with cGMP formation. Supportive of the contingent involvement of cGMP is the finding that N1E-115 cells will hyperpolarize when cGMP is added to the medium (98).

The hypothesis that cGMP is the mediator of neuronal muscarinic-linked ionic events via direct activation of the cyclase by Ca^{+2} passing in through channels is complicated somewhat by the recent finding that N1E-115 cells loaded with the Ca^{+2}-sensitive photoprotein aequorin (101) do not emit light when stimulated with carbachol (E. Richelson, R. M. Snider, M. McKinney, C. Forray, submitted for publication), indicating the absence of a rise in intracellular free Ca^{+2}. The preloaded cells will emit light when stimulated by high K^+ or with the ionophore X537A, however. Ca^{+2} influx into the cytosolic pool then may not be a consequence of neuronal muscarinic stimulation (105). A messenger other than Ca^{+2} may be interposed between receptor and cyclase (see below). This additional step may explain the odd voltage-dependence of the N1E-115 delayed K^+ channel (95, 97).

If cGMP formation is not involved in ionic events, one must consider alternative functions in neural tissue; it is interesting to note in this context that

cGMP has been shown to be involved in memory at the cellular level in sympathetic ganglia (103), although in mammals it has not yet been possible to show a direct link between cGMP and changes in membrane conductance (104). Cylic GMP thus may not in general mediate the voltage changes in nervous tissue typically seen with muscarinic agents, but in both cortical neurons and N1E-115 cells it appears that a rise in intracellular cGMP concentration does by an obscure mechanism lead to membrane conductance changes.

Biochemical Responses

PHOSPHOLIPID METABOLISM Acetylcholine, acting at muscarinic receptors, is one of the many agents that can stimulate the phosphatidylinositol (PI) cycle (22–24). Several lines of evidence support a role for the PI cycle in neurotransmitter action in the CNS (24); much of this evidence concerns muscarinic action. Although the metabolism of PI and the less prevalent polyphosphoinositides (PPI) is stimulated by brain muscarinic receptors and Ca^{+2} seems to be required for this, it is not yet certain whether neurotransmitter-stimulated Ca^{+2} influx is the required Ca^{+2} event. This aspect of the hypothesis of the PI cycle (Ca^{+2} gating) remains unsettled.

Muscarinic-mediated PI metabolism in brain tissue has been demonstrated in vivo and in vitro (with synaptosomes or slices) by the incorporation of ^{32}P-phosphoric acid into PI, PPI, and phosphatidic acid (PA), and by release of [^{3}H]*myo*inositol phosphate from PI (24). In a typical study, carbachol was shown to stimulate the release of inositol-phosphate(s) 5.4–8.3× over basal in slices of cortex, striatum, hypothalamus, and hippocampus (24). Removal of the predominant cholinergic input to the hippocampus by a fornix lesion does not affect muscarinic-mediated PI turnover in hippocampal synaptosomes (105), while direct lesion of intrinsic hippocampal neurons with the neurotoxin ibotenic acid does reduce synaptosomal PI turnover (106). This is good evidence that synaptosomal PI turnover is located post-synaptically.

The agonist stimulation of the synaptosomal PI cycle does not require Ca^{+2} in the medium, but added EGTA will block it (107). This has been interpreted to mean that an endogenous store of Ca^{+2}, perhaps plasma membrane–bound, is available as a source for the effect of the neurotransmitter. The divalent cation ionophore A23187 will stimulate a similar synaptosomal PI turnover and this is not blocked by atropine (108). Submaximal levels of A23187 and acetylcholine added together cause a synergistic effect. It has thus been supposed that calcium influx occurs with both types of stimulation. The hypothesis of a common pathway of Ca^{+2} influx preceding the PI cycle is supported by the blockade of the effect of both agents by EGTA. However, although influx of Ca^{+2} as caused by A23187 is sufficient to elicit PI labeling, it is not necessary to suppose that the receptor causes influx. The muscarinic receptor may or may not use the same mechanism to stimulate the cycle. This can be seen in the

situation cited above, where the cGMP response of N1E-115 cells can be elicited by both ionophore X537A and by carbachol, yet Ca^{+2} transport can be measured only with the ionophore. The mechanism of neurotransmitter stimulation of the synaptosomal PI cycle then may also not require transport of Ca^{+2} to the inside in the same way an ionophore does.

Agranoff et al have recently refined their study of the synaptosomal muscarinic receptor by comparing agonist occupancy curves with PI dose-response curves (63). Full but not partial muscarinic agonists bound to two sites, with the majority of sites (65%) in the low-affinity conformation. Carbachol's dose-response curve, however, was nearly monophasic with an ED_{50} of 109 μM, quite close to the K_D for the low-affinity site (77 μM). The conclusion is that the low-affinity agonist site of cerebral cortex mediates the synaptosomal PI turnover. By analogy with the hippocampal studies cited above, these findings in the cortex probably reflect a post-synaptic localization for the response.

Mn^{+2} is generally regarded as a Ca^{+2}-channel blocker in neural tissue. However, it modulates the PI cycle in synaptosomes (109) by increasing incorporation of inositol into PI. Mn^{+2} will inhibit the PI labeling by [^{3}H]inositol evoked by the muscarinic receptor. The mechanism of Mn^{+2} stimulation may involve a stimulation of the PI cycle by stimulating PI synthesis (24, 109). This is one example of a mechanism of stimulating the PI cycle that probably involves neither Ca^{+2} influx nor a stimulation of PI turnover. Mn^{+2} alone transiently increases cGMP formation in N1E-115 cells and with prolonged incubation it will inhibit carbachol-mediated cGMP formation (80, 81). This is an interesting parallel with the effects of Mn^{+2} on the synaptosomal PI cycle, suggesting that Mn^{+2} and the neurotransmitter may activate a common pathway for the stimulation of cGMP synthesis. One implication is that the muscarinic receptor may mediate the cGMP response by stimulating the PI cycle, and this is supported by the recent finding that N1E-115 muscarinic receptors mediate PI turnover (28).

As with the cGMP response, the PI turnover response of N1E-115 cells desensitizes (28). Ca^{+2} in the medium is not required for carbachol to stimulate labeling of PI in N1E-115 cells. As Ca^{+2} can be absent from the medium and the cGMP response will also still desensitize, this is a feature common to these two responses. The half-maximal PI response of N1E-115 cells occurs at 10 μM. This implies that the N1E-115 PI cycle and cGMP response are both stimulated by the low-affinity receptor and that desensitization of the two responses might be by the same mechanism, perhaps at a point in the PI cycle.

In smooth muscle cells, where there is a Ca^{+2}-dependent muscarinic-mediated cGMP response, exogenous PA will cause contraction with a time course similar to that of carbachol (111). PA has been proposed as the mediator of the presynaptic Ca^{+2} influx that causes neurotransmitter release (112).

Studies such as these implicate PA as a Ca^{+2} ionophore. PA could then be a candidate link between the PI cycle and the other responses of N1E-115 if it acts to carry Ca^{+2} inside. Exogenous PA will cause $^{45}Ca^{+2}$ uptake and cGMP formation in N1E-115 cells (110), and it will evoke the emission of light when the cells are preloaded with the photoprotein aequorin (R. M. Snider, personal communication). However, the guanylate cyclase is a soluble enzyme in N1E-115 cells (113) and is probably not directly activated by Ca^{+2}, so there is a question as to what intracellular agent stimulates the cyclase.

In platelets, the stimulation of the PI cycle by thrombin causes PI to be metabolized by a phospholipase C (PLC) and a phospholipase A_2 (PLA_2) (114). Diacylglycerol is formed, which is phosphorylated to PA, while arachidonic acid is released. The arachidonate release is probably dependent upon the operation of the PI cycle because protease inhibitors, which inhibit the PLC, block the release and metabolism of arachidonate (115). Since the PLA_2 is a Ca^{+2}-dependent enzyme, one possible mode of its activation by the PI cycle could be by transport of Ca^{+2} into its vicinity, perhaps by chelation to headgroups of acidic phospholipids (e.g. PA), which could be generated locally by receptor activation of the PI cycle. A complex of Ca^{+2}-PA is electrically neutral and although phospholipids do not normally flip-flop, a Ca^{+2} electrochemical gradient can apparently supply the necessary energy for this in smooth muscle (111) and in synaptosomes (112) in a Pressman chamber (116). Arachidonic acid metabolism has been recently implicated in the N1E-115 neurotransmitter-mediated cGMP response: thrombin will stimulate cGMP-formation in these cells with a Ca^{+2}-dependence and a time course similar to that of the neurotransmitter (117).

CYCLIC NUCLEOTIDE RESPONSES

Muscarinic stimulation of cyclic GMP in brain tissue and neuroblastoma cells The formation of cGMP, discussed above in several contexts, is a common response to muscarinic activation (14). By analogy with what is known of cAMP, a second messenger role for cGMP has been postulated (118). Protein phosphorylation by cGMP-dependent protein kinases can be demonstrated in smooth muscle (119); cGMP-dependent protein phosphorylation has been shown to occur in mammalian brain (where a 23,000-dalton protein is phosphorylated), but a link to the muscarinic receptor has not been shown (120). In primary cultures of rat cerebellum, long-term exposure to carbachol causes phosphorylation of proteins (121) but we do not know whether this is mediated by cGMP or not. cGMP is certainly a good indicator of neuronal muscarinic activation, but specifically what types of physiological responses it mediates is unclear. When its level is increased in cortical neurons or in N1E-115 cells it can lead to changes in membrane resistence but by unknown mechanisms.

Levels of cGMP in various areas of the brain are elevated with systemic administration of oxotremorine, a muscarinic agonist (12). Immunohistochemical reactions to cGMP in nervous tissue have shown that it is present in neurons and glial cells in the caudate-putamen (122), in neurons and glial cells in the cerebellum (123), and in neurons of the superior cervical ganglion (8). Slices of brain tissue form cGMP when incubated with muscarinic agonists (9, 10), though the degree of response is never very large, presumably because only a small fraction of the neurons in the slice are cholinoceptive.

The dose-response curves for agonists in slices of rat striatum have Hill slopes closer to unity than their occupancy curves, and the ED_{50} for carbachol and arecholine are near their dissociation constants at the low-affinity receptor (9). Ca^{+2} at a physiological level is required and the Ca^{+2} dependence appears similar to that for muscarinic responses in N1E-115 cells. In this study of the striatum, cAMP was also elevated by muscarinic agonists and the formation of both nucleotides was blocked by atropine. It is not known whether the muscarinic stimulation of cGMP was direct or involved interneurons, but the cAMP response could also be blocked by a dopamine antagonist, indicating that cAMP was formed after dopamine release stimulated by the muscarinic agonist. This illustrates some of the complexities in interpreting the results of experiments with brain slices. In a study of the septo-hippocampal pathway, electrical stimulation of the medial septum caused elevated cGMP levels in the hippocampus, but this was not blocked by systemic administration of scopolamine (124). As there is a cholinergic component to this pathway, these data do not support a post-synaptic role for cGMP in the brain, which is demonstrable in the superior cervical ganglion (8). Thus, the evaluation of a link between the muscarinic receptor and cGMP in the brain is not simple.

N1E-115 cells display many of the properties of neurons, including the possession of neurotransmitter receptors, the formation of cyclic nucleotides with stimulation of these receptors, and the possession of voltage-sensitive ion channels that can operate action potentials (125). N1E-115 muscarinic receptors mediate a marked rise in intracellular cGMP and an inhibition of neurotransmitter-stimulated cAMP formation (13, 35). NG108–15 hybrid neuroblastoma-glioma cells have muscarinic receptors that inhibit cAMP formation and are capable of acting on a guanine nucleotide binding protein (15, 18). Thus, these two cell lines provide useful model systems to ask questions about cyclic nucleotide responses to muscarinic activation that pertain to responses in the brain.

Coupling and cyclic GMP response in N1E-115 cells Two types of agonist binding sites exist in membranes prepared from homogenates of N1E-115 cells, with dissociation constants for carbachol of 50 μM (K_L) and 0.1 μM (K_H) (53). The dose-response curve for carbachol-mediated cGMP formation via the

muscarinic receptor has an ED_{50} at about 70 μM, not too far from the K_L (27, 34, 35). The Hill coefficient for muscarinic stimulation of cGMP is of unity or greater value, while the agonist binding Hill coefficient is always less than unity due to the presence of multiple sites. The ED_{50} for agonist desensitization of the receptor is also close to the ED_{50} for cGMP stimulation (126). All this taken together suggests that two sites are involved in binding, while only one, probably the low-affinity site, is involved in the stimulation of cGMP formation and its desensitization. This seems to be a parallel with the synaptosomal PI response discussed above.

Increasing the Ca^{+2} concentration from 1.8 mM to 10 mM increases the potency of carbachol's cGMP stimulation while not affecting its binding (79); this presumably reflects an action of the ion at the putative effector, which mediates the stimulation with an absolute requirement for Ca^{+2}. The lanthanides, which are classical Ca^{+2} antagonists, block this effector (79), possibly by competition with Ca^{+2} at sites of its action, because increasing the Ca^{+2} concentration can overcome the depression of V_{MAX} by the lanthanides. Lanthanides also decrease the ED_{50} for carbachol, suggesting that these ions have a separate effect on receptor-effector coupling. As discussed above, Mn^{+2} can induce cGMP formation in the presence of Ca^{+2}, with a time course similar to that of the neurotransmitters (80). This is probably an action on the coupling mechanism, because Mn^{+2} does not affect [^{3}H]-QNB binding to the N1E-115 receptor and probably does not enter the cell. Verapamil will block both muscarinic- and Mn^{+2}-mediated cGMP formation, apparently by blockade of Ca^{+2}-channels, and this agent will displace [^{3}H]-QNB from the receptor (81). These data taken together support a concept of muscarinic receptors as physically coupled to Ca^{+2}-effectors. These effectors could be Ca^{+2}-channels, but because neurotransmitter-stimulated Ca^{+2} influx is not observable, it is preferable to refer to them as Ca^{+2}-effectors. The concept of precoupled receptors (i.e. that they exist coupled in both the basal and stimulated states) is also supported by an experiment that showed that desensitization of the muscarinic and histamine receptors has an additive inhibition on Mn^{+2}-mediated cGMP formation (80). Thus, the mobile receptor hypothesis (127) (which presumes that receptors are uncoupled in the basal state) may not apply to the initial cGMP response in N1E-115 cells. These cells might change the proportion of pre-coupled receptors in the regulation of this response, however, and in this sense the latter hypothesis may apply (126). The coexistence of precoupled and uncoupled N1E-115 receptors may also explain multiple agonist binding sites if coupling to a channel affects the conformation of the receptor (54, 78). This is the more probable situation if binding studies reveal a constant ratio of sites with a range of full agonists, as is observed in cortical membranes (52). This would mean that the receptors are pre-coupled. However, it has not yet been

rigorously shown that binding in homogenates accurately reflects the conformations of the receptor in intact cells.

Calcium translocation and the cyclic GMP response in N1E-115 cells All the agents that stimulate cGMP formation in N1E-115 cells require Ca^{+2} to be present in the medium. However, as noted above, Ca^{+2} transport is not measurable during neurotransmitter stimulation of cGMP formation. Macroscopic Ca^{+2} translocation thus does not occur with the neurotransmitters. Yet Ca^{+2} must be present on the outside of the cells for cGMP to be formed intracellularly, and Ca^{+2} is somehow involved in the muscarinic receptor-effector coupling mechanism. It appears that subsequent to agonist binding, a transmembrane event not involving Ca^{+2} flux into the cytosol per se is catalyzed by the presence of Ca^{+2}. This implies the involvement of Ca^{+2} located in the plasma membrane pool that is accessible to extracellular free Ca^{+2}. Such a concept of Ca^{+2} movement has been proposed for the "filling phase" of muscarinic receptor-stimulated Ca^{+2} fluxes in parotid acinar cells (128).

High K^+ (110 mM) in the medium, X537A, and PA all translocate Ca^{+2} inside and stimulate cGMP formation with a profile containing a rise to a peak and a return to basal levels. Ca^{+2} influx with these agents is very rapid but the time course of the subsequent cGMP formation is slower, even slower than that seen with carbachol or histamine (E. Richelson, R. M. Snider, M. McKinney, C. Forray, submitted for publication). First, this suggests that the calcium dependence of the action of the neurotransmitter to stimulate cGMP formation is somehow vectorial. Second, it suggests that an additional time-dependent process is involved. As was suggested above, Ca^{+2} may be chelated by head groups of phospholipid moities locally generated by the PI cycle, possibly PA, which then bind to and activate proteins within the membrane or activate an internal membrane-associated protein such as the enzyme phospholipase A_2 or diglyceride lipase, which releases another messenger. This would satisfy the vectorial and possibly the temporal constraint. Another possibility is that a Ca^{+2}-PA complex is the effective substrate for the PLA_2. The metabolism of PA by the muscarinic-stimulated PI cycle in N1E-115 cells has recently been shown (28), and its independence of Ca^{+2} might be taken as supportive of a role for a PI cycle product in some type of neurotransmitter-linked Ca^{+2} translocation. In any case, because of lack of evidence of transport into the cytosol, such a mechanism requires that the internal Ca^{+2} concentration does not change, so Ca^{+2} remains in a bound state or is released in minute quantities. It is thus hypothesized that the effector coupled to the receptor uses Ca^{+2} to mediate the release of another messenger (possibly arachidonic acid; see below) that can enter the cytosol and that effects stimulation of guanylate cyclase (102). The function of the effector (which may be the PI cycle) is to bring Ca^{+2} into the

vicinity of this "activator," which actually releases the messenger (see Figure 1).

According to this hypothesis, Mn^{+2} is able to bypass the receptor and, by acting on the effector, directly trigger the Ca^{+2}-dependent membrane event leading to cGMP formation. This action is not specific to the muscarinic receptor; Mn^{+2} also activates the effector of the histamine receptor (80, 81). As Mn^{+2} is able to modulate the PI cycle in synaptosomes, Mn^{+2} may modulate the PI cycle in N1E-115 cells, which then leads to translocation of Ca^{+2} into the vicinity of the activator, followed by the stimulation of cGMP formation in these cells.

Implications from the time course of cyclic GMP formation Upon receptor-linked stimulation of cGMP formation in N1E-115 cells, the levels of the cyclic nucleotide rise rapidly, typically to 5- to 15-fold over basal levels (13, 35). This is followed by a rapid return of cGMP levels to the basal value. The time to the peak varies with the stimulant (carbachol, histamine, thrombin) but usually lasts between 30–60 seconds. This temporal profile has been observed in other systems.

Considering muscarinic receptor stimulation, the climb to the peak is the result of turning on the membrane event R→E→A (see Figure 1). This peak

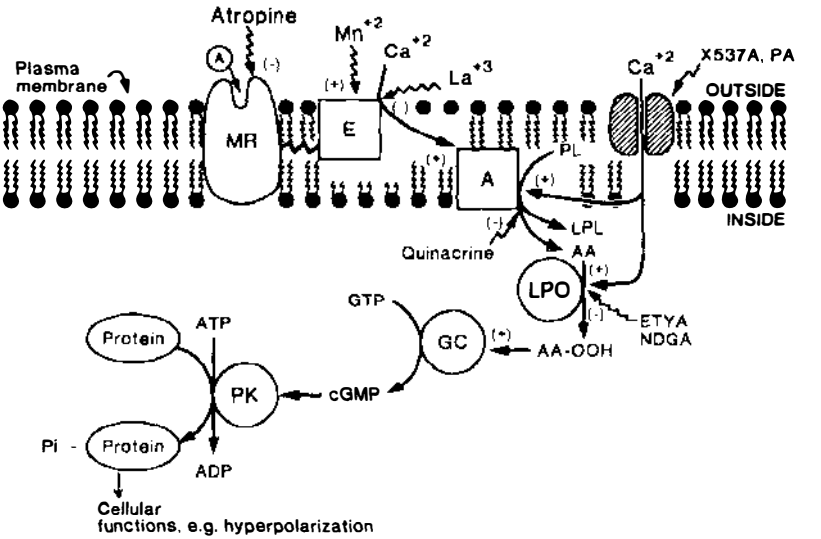

Figure 1 Hypothetical scheme for molecular events involved in muscarinic-receptor mediated cGMP response in neurons. MR: muscarinic receptor; Ⓐ: agonist; [A]: activator; [E]: effector; X537A: ionophore; PA: phosphatidic acid; PL: phospholipids; LPL: lysophospholipids; AA: arachidonic acid; ETYA: eicosatetraynoic acid; NDGA: nordihydroguairetic acid; AA-OOH: hydroperoxide of arachidonic acid; GC: guanylate cyclase; PK: protein kinase. For explanation, see text.

concentration is hyperbolically dependent upon the neurotransmitter concentration and is thus proportional to occupancy of some receptor subtype. Therefore, the neurotransmitter, by its binding to the receptor, effects the rapid stimulation of the guanylate cyclase to a high and constant velocity. This distinctive temporal profile implies that the guanylate cyclase is an allosteric enzyme and that its activating ligand is released subsequent to binding of the agonist. The sharpness of the peak at 30 seconds means that a turning-off event occurs: near the peak the activating process ceases and the removal of cGMP begins or takes over. In the absence of phosphodiesterase inhibition the cGMP levels decline hyperbolically. Isobutylmethylxanthine, a phosphodiesterase inhibitor, flattens the decline, indicating that phosphodiesterase is involved in the removal process (13). Lowering the temperature also prolongs the decline and shifts the peak to the right; thus, either the phosphodiesterase is temperature-sensitive or the turning-off process is a membrane event dependent on membrane fluidity (87).

The profiles of cGMP levels during stimulation of cGMP by K^+, Mn^{+2}, and PA also contain the rapidly declining phases (80, 96, 110). Thus, because these agents probably do not activate receptors directly, the turn-off event must be downstream of the receptor.

Activating mechanism By a calcium-dependent process, thrombin, a PLA_2 stimulant in platelets, stimulates cGMP formation in N1E-115 cells (117). This implicates membrane-derived fatty acids, possibly arachidonic acid, in this response. Quinacrine, an inhibitor of PLA_2, blocks thrombin-, histamine-, and carbachol-mediated cGMP formation in these cells (117; M. McKinney, R. M. Snider, E. Richelson, unpublished information). Lipoxygenase (LPO) inhibition with nordihydroguairetic acid or eicostetraynoic acid also blocks cGMP formation; it is unlikely that these agents have a direct effect on the receptor. Indomethacin, which blocks the cycloxygenase that synthesizes prostaglandins, has no effect on carbachol- or thrombin-mediated cGMP formation. The effects of the inhibitors suggest that the receptor causes the release of arachidonic acid and that the lipoxygenase produces a metabolite that stimulates the guanylate cyclase.

The guanylate cyclase of guinea pig myometrium can be activated by arachidonate metabolites in broken cell preparations (22), and the guanylate cyclase in rat ductus deferens is stimulated by the muscarinic receptor through a lipoxygenase metabolic pathway (25). Guanylate cyclase in other tissues can be activated by unsaturated fatty acids and their derivatives (129, 130). Recently the platelet guanylate cyclase has been shown to be activated by membrane-derived arachidonate (130a). Since the initial product formed by lipoxygenase is a hydroperoxide and since hydroperoxide metabolites of arachidonate were most effective in stimulating guanylate cyclase in most of the above studies, it is expected that the activating agent in N1E-115 will turn out to be a transient

arachidonate hydroperoxide. Since the PI cycle is stimulated in N1E-115 cells by the muscarinic receptor and because this cycle has been implicated in the activation of PLA_2 in other systems, it is reasonable next to examine the requirement for PI turnover in cGMP responses and determine if the activation of PLA_2 in N1E-115 cells is the mechanism of generating an intracellular messenger for the muscarinic receptor. It is likely that the PLA_2 and lipoxygenase of N1E-115 cells are Ca^{+2}-dependent, as they are in other systems (131, 132); exactly how Ca^{+2} reaches these enzymes to activate them must await detailed biochemical studies.

Turnoff of cGMP formation could be due to a cessation of PLA_2 activity. The cessation of activity could be due to feedback inhibition of free arachidonate or one of its metabolites on the Ca^{+2}-transport mechanism or on PLA_2. Alternatively, PLA_2 may turn off simply because of a local depletion of its substrate or because of product inhibition by the lyso-phospholipids.

Cyclic AMP and a role for the high-affinity muscarinic binding site of N1E-115 cells The possibility of a physiologic response mediated by the high-affinity receptor is supported by the muscarinic inhibition of prostaglandin E_1-stimulated adenylate cyclase activity in N1E-115 cells analogous to that seen in NG108–15 cells. In experiments in which the same cells were assayed for both stimulation of cGMP and inhibition of prostaglandin E_1-stimulated cAMP formation, there was a separation of the ED_{50}'s for these two responses by nearly two orders of magnitude (62). The ED_{50}'s for the effects are 50 μM for cGMP stimulation and 1 μM for cAMP inhibition. These are remarkably close to the dissociation constants for the two sites. The receptor-mediated inhibition of adenylate cyclase in NG108–15 by opiates, α-adrenergic agents, and muscarinic agents is likely to be by stimulation of a GTPase activity that results in the dissociation of the guanyl nucleotide protein from the adenylate cyclase (133). Muscarinic receptor interaction with a guanyl nucleotide protein is suggested by the effects of muscarinic agonists on the β-adrenergic-receptor-mediated cAMP formation in canine cardiac membranes (134) and by the effects of GTP on brain and heart muscarinic receptor binding (40, 41, 55, 56). Thus, for N1E-115 muscarinic receptors the high-affinity agonist subtype could be mediating an effect on the coupling of the prostaglandin E_1 receptor to guanine nucleotide proteins by causing hydrolysis of GTP and subsequent decoupling of the putative guanyl nucleotide protein from adenylate cyclase. The presence of isobutylmethylxanthine does not prevent the muscarinic inhibition, which means that hydrolysis by phosphodiesterase is not stimulated via the muscarinic receptor to cause the cAMP reduction (S. Stenstrom, E. Richelson, unpublished information). Since prostaglandin E_1 will also stimulate cGMP formation in N1E-115 cells and this is additive to carbachol's

stimulation (13), a simple shuttling mechanism of a putative guanine nucleotide regulatory protein is difficult to envision.

Short-term desensitization of neuroblastoma muscarinic responses Attenuation of N1E-115 cGMP and PI responses occurs upon prolonged incubation of these cells with muscarinic agonists (27, 28). Because these effects occur in minutes, it is referred to as short-term desensitization. With desensitization, the dose-response curve for carbachol-mediated cGMP formation shifts to the right and the maximum is depressed, with increasing times of preincubation with the agonist (27). If the cells are then incubated without agonist present, they will gradually become increasingly responsive to another challenge. These processes will not occur below 21°C, which probably means that membrane fluidity is required (87). Desensitization will occur without Ca^{+2} ions in the medium, indicating that cGMP formation is not required for it to occur, which is further support for the hypothesis that desensitization is a membrane event. Additionally, the process is dose-dependent (126). A candidate site for the event(s) then is the receptor itself. Antagonist binding to the muscarinic receptor of N1E-115 cells is not changed by desensitization, but recently it has been shown that the binding of agonists is affected by desensitization (62). The dissociation constants for all three agonist sites are increased during desensitization, perhaps accounting for the rightward shift in dose-response curves.

Histamine H_1 receptors and muscarinic receptors of N1E-115 cells do not cross-desensitize more than 15%; this specific desensitization is evidence for a change in the receptor protein or a protein coupled to it. Because these receptors both trigger cGMP responses in a Ca^{+2}-dependent fashion, it is possible that they use the same types of effectors. Mn^{+2}, an agent which can mediate cGMP formation without a neurotransmitter present, will inhibit histamine- and carbachol-mediated cGMP formation. This inhibition by Mn^{+2} is similar to the attenuation seen in desensitization. If the cells are desensitized to either histamine or carbachol, the cGMP response mediated by Mn^{+2} is reduced by about half. Mn^{+2} does not affect receptor binding, as observed with [^{3}H]-QNB. The site of the action of Mn^{+2} is likely then to be on the effector to which receptors couple. The Ca^{+2} dependence of Mn^{+2}-mediated cGMP formation also supports this. Additionally, and most importantly, the simultaneous desensitization of these two receptors has almost additive inhibitory effects on the Mn^{+2} stimulation (80). This strongly suggests that each receptor is coupled to a dedicated effector and that desensitization occurs not only at the receptor but also at the effector or at the mechanism of their coupling. What kind of change occurs in the effector or on the coupling mechanism cannot be addressed until more is understood about what role Ca^{+2} ions have in generating the intracellu-

lar messenger; i.e. how the effector functions. Initial effort might be best directed at discerning if the PI cycle and arachidonic acid metabolism are involved. It should be kept in mind that the PI response to the muscarinic receptor of N1E-115 cells also displays short-term desensitization (28).

Resensitization is a slower process and is not dependent on the agonist concentration used to desensitize (87, 127). The dose-response curves for resensitized cells shift inversely to the direction of shift with desensitization, suggesting that the process is a simple reversal. Its slowness suggests continuous enzymatic action; a conformational change might be expected to occur more quickly. Though one may suspect changes in covalent bonds, such as disulfide bond formation, there is no direct evidence as yet of this phenomenon. Sulfhydryl agents do affect muscarinic receptor binding in brain homogenates; some have suggested that the muscarinic receptor shifts between agonist states with N-ethylmaleimide treatment or with the transitional metals.

The muscarinic receptors of NG108–15 neuroblastoma-glioma cells also desensitize; they become less able to inhibit the prostaglandin E_1-mediated cAMP response (29). This desensitization is specific to the muscarinic receptor, since α-adrenergic receptors or opiate receptors can still inhibit prostaglandin E_1-mediated cAMP formation. The GTP coupling proteins, extracted from desensitized NG108-15, were still able to stimulate cAMP in another, coupling-protein-deficient cell, so it is likely that it is the receptor or its ability to act on the GTP protein that has been affected by desensitization. Somehow desensitization has caused the receptor to lose its effect on the GTPase. It is not yet known whether desensitization occurs with the N1E-115 muscarinic receptor-mediated inhibition of prostaglandin E_1-stimulated cAMP formation.

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