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AGONIST-INDUCED MUSCARINIC CHOLINERGIC RECEPTOR INTERNALIZATION, RECYCLING AND DEGRADATION IN CULTURED NEURONAL CELLS

CELLULAR MECHANISMS AND ROLE IN DESENSITIZATION

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Abstract—Short-term incubation of intact neuronal cells with muscarinic cholinergic agonists resulted in a rapid decrease of the specific binding of [^{3}H]methylscopolamine to cell surface receptors indicative of receptor internalization. The agonists induced the internalization of both the muscarinic receptor subtypes coupled to adenylyl cyclase and those coupled to phosphoinositide turnover. Receptor internalization, which was inhibited at 0–4° and by depletion of intracellular K^+, is thought to occur through coated pits formation and was rapidly reversible. Receptor recycling did not imply protein synthesis. Down-regulation of muscarinic receptors occurred slowly in the presence of agonists, needed intact cytoskeleton (demonstrated by the inhibitory effect of colchicine) and involved lysosomal activity. Both receptor internalization and down-regulation were prevented by muscarinic receptor antagonists. Receptor internalization and down-regulation are agonist-induced cellular mechanisms that with receptor phosphorylation and uncoupling, may induce desensitization. These processes may contribute to complex intracellular regulatory processes and may be involved in some of the long-term effects of neurotransmitters (mainly neuropeptides and growth hormones) or drugs.

Key words: desensitization, internalization, down-regulation, muscarinic receptors, [^{3}H]methylscopolamine, cell culture

Desensitization significantly limits the clinical use of many pharmacological agents and could play a role in the development of tolerance and dependence to several drugs such as opiates and benzodiazepines. However, the slow process which leads to tolerance and dependence has to be distinguished from desensitization because most desensitization processes occur within seconds or minutes and even the down-regulation of receptors occurs within a few hours whereas slowly developing sensitivity changes, which are thought to lead to tolerance and dependence, generally require several days or weeks to reach their maximum and disappear equally slowly when the stimulus is removed. The desensitization mechanisms may differ from one system to the other but, if we consider the large family of G-protein coupled receptors, we have to distinguish molecular processes from cellular mechanisms which include receptor internalization and increased rates of degradation. Different subtypes of muscarinic receptors are present in cultured neurons, they may be identified at the cell surface of intact cells and several agonists and antagonists are available for their study. For these reasons, we will focus in this report on the agonist-induced muscarinic receptor regulation at a cellular level in cultured neuronal cells.

Ligand binding to cell surface muscarinic receptors in cultured neuronal cells

Muscarinic receptors have been characterized in several neuronal cell clones, such as mouse neuroblastoma N1E-115 cells [1–3], mouse neuroblastoma × rat glioma NG108-15 hybrid cells [1, 4, 5], human neuroblastoma IMR-32 and SH-SY5Y cells [6] and rat pheochromocytoma PC12 cells [7]. Muscarinic receptors have also been described in primary cultures of rat neuronal cells [8, 9]. Most of the labeled ligands available for specific binding to muscarinic receptors are lipophilic, such as [^{3}H]QNB† [10–12], [^{3}H]dexetimide (*d*-benzetimide) [13, 14] or [^{3}H]pirenzepine [15–17] and they do not discriminate between cell-surface receptors and receptors localized within the cells. One ligand, [^{3}H]-*N*-methylscopolamine [18, 19] is less lipophilic than the others, and for this reason binds to cell-surface muscarinic receptors only and does not gain access to receptors localized in intracellular vesicles [9, 20]. Moreover, this ligand is not accumulated in intracellular compartments such as lysosomes where most labeled drugs are concentrated because of the acidic lysosomal pH [21, 22]. We used [^{3}H]-*N*-methylscopolamine to study the disappearance of cell surface receptors and their recycling in different cultured neuronal cells.

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† Abbreviations: QNB, quinuclidinyl benzylate; βARK, β-adrenergic receptor kinase; DAG, diacylglycerol; PI, phosphoinositide; PMA, phorbol-myristate-acetate; NEM, *N*-ethylmaleimide; EGF, epidermal growth factor.

Muscarinic receptor heterogeneity, which was initially suggested by the complex binding profile of the antagonist pirenzepine, has now been clearly demonstrated. At least five different genes encode muscarinic receptor subtypes, which all belong to the G-protein coupled receptors family [23–25]. Most authors consider that muscarinic receptors in NG108-15 neuroblastoma × glioma cells are coupled to adenylyl cyclase (m_2 or m_4 subtype) whereas muscarinic receptors in N1E-115 neuroblastoma cells stimulate phosphoinositide breakdown (m_1 subtype). Muscarinic receptors of the m_1, m_3 or m_5 subtypes, coupled to phosphoinositide turnover, are also found in other cells including human neuroblastoma IMR-32 and SH-SY5Y cells and rat cerebellar granule cells, whereas m_2 or m_4 receptors, coupled to adenylyl cyclase, predominate in human astrocytoma 1321-N1 cells and PC12 pheochromocytoma cells [26–29]. Muscarinic receptors that are linked to the different second messengers may coexist in some cell types.

Cholinergic agonists interact with at least two distinct populations of sites; high and low affinity sites [10, 30–32]. Guanine nucleotides induce interconversion of agonist high-affinity sites to the low-affinity state [10, 33–35]. In NG108-15, N1E-115 and primary cultured neuronal cells, there is a high affinity and saturable binding of [^{3}H]QNB to a total particulate fraction [22] and of [^{3}H]-*N*-methylscopolamine to intact cells [9]. In these different cell types and in the absence of cholinergic stimulation, the majority of the receptors are located at the cell surface and among these receptors, binding studies performed at 4°, using agonists as displacers of the labeled ligands, revealed that about 80% of the receptors at the cell surface were present in a low affinity state for the agonists.

Muscarinic receptor regulation in cultured neurons

A reduction in responsiveness of neuronal cells to cholinergic agonists (desensitization) could be brought about by several mechanisms: (1) structural changes of the receptor involving its phosphorylation; (2) uncoupling of the receptor from its associated G-protein and/or effector (disruption of the coupling might occur through receptor phosphorylation); (3) internalization of the receptor and therefore inaccessibility of the agonist to the receptor; (4) decrease in receptor number (down-regulation) corresponding to degradation or reduced synthesis, which might follow agonist-induced receptor internalization.

Structural changes involved in receptor desensitization. Possible role of receptor phosphorylation

Receptor phosphorylation has been shown to play a role in the mechanism of β_2-adrenergic receptor desensitization [36, 37] but this is less clear for other receptors including acetylcholine muscarinic receptors. Phosphorylation of brain synaptic membrane fragments was reported to induce a decrease of [^{3}H]QNB specific binding to muscarinic receptors [38, 39]. This decrease was higher in the cerebellum than in the cerebral cortex but did not exceed 30% of control values and could be reversed by a protein phosphatase [40]. Increased phosphorylation of some membrane proteins was observed after exposure of cultured neurons to muscarinic agonists [8]. Muscarinic receptors contain potential sites of phosphorylation by a cAMP-dependent protein kinase [41]. Purified muscarinic receptors from rat brain, at least some of them, are a substrate for cAMP-dependent protein kinase and phosphorylation of these receptors reduces their ability to bind [^{3}H]-QNB [42]. In chick heart, the level of phosphorylation of purified muscarinic cholinergic receptors (m_2-subtype) was found to be 10 times higher after desensitization following exposure to muscarinic agonists [43].

Agonist-induced phosphorylation and uncoupling of the β_2-adrenergic receptors is known to result from the activation of either a cAMP-dependent kinase (protein kinase A) or a βARK. At low agonist concentrations, protein kinase A is activated, which phosphorylates one or two sites adjacent to the region of the receptor involved in the coupling with the α subunit of Gs (αs) protein. This phosphorylation disrupts the coupling. This protein kinase A can also phosphorylate other activated receptors, including some muscarinic subtypes, and therefore may play a role in their desensitization. At high concentrations of agonists, the rapid homologous desentization may occur through phosphorylation of the receptor by the cAMP-indepedent kinase, βARK [44]. βARK is a kinase which phosphorylates the β-adrenergic receptors [45, 46] and also other G-protein coupled receptors including α2-adrenergic receptors and heart muscarinic (m_2) receptors. Therefore, homologous desensitization of the m_2-muscarinic receptor subtype might result from a mechanism similar to that of the β_2-adrenergic receptors. In this way, agonist-induced phosphorylation and desensitization of human m_2 (but not m_1) muscarinic receptors expressed in Sf9 insect cells has been recently reported [47]. In these cells, pertussis toxin suppressed the interaction between m_2-receptors and G-proteins without changing the ability of carbachol to induce receptor phosphorylation, suggesting that G-proteins or G-protein-activated signals were not necessary for agonist-induced phosphorylation of the receptors.

Protein kinase C, which is activated by muscarinic (m_1, m_3, m_5) receptor occupancy might also mediate receptor phosphorylation. The breakdown of phosphatidylinositol bisphosphate induced by muscarinic agonists generates DAG, an endogenous activator of protein kinase C [48, 49]. The role of protein kinase C in desensitization has therefore been studied in N1E-115 cells using phorbol esters that activate protein kinase C by substituting for the endogenous DAG. Phorbol esters such as PMA which activate protein kinase C are reported to promote rapidly the translocation of protein kinase C from the cytosol to the cellular membrane. This translocation has been shown in several systems to correlate with the activation of protein kinase C [48, 50, 51]. PMA was shown to desensitize the muscarinic receptor-mediated stimulation of cGMP synthesis that is mediated through activation of the muscarinic receptor (m_1 subtype) in N1E-115 cells [52–54]. PMA induced a desensitization which is protein kinase C-dependent but without receptor internalization, whereas the agonists induced a protein

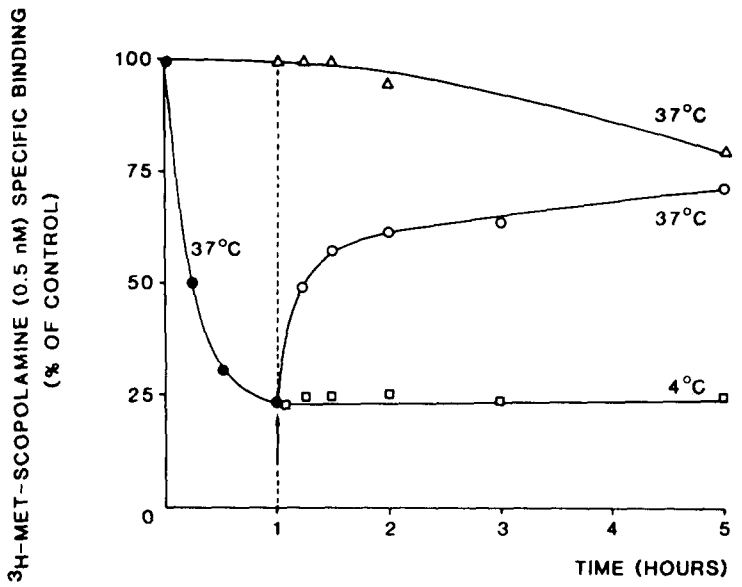

Fig. 1. Time course of carbachol-induced effect on muscarinic receptors. Intact neurons (cerebellar cultured cells) were incubated with carbachol (0.5 mM) at 37° up to 1 hr (●). Cells were then washed to remove carbachol and were further incubated at 37° (○) or 4° (□). The specific binding was measured in intact cells with 0.5 nm [^{3}H]-*N*-methylscopolamine (●, ○, □) or in cell homogenate with 1 nM [^{3}H]-QNB (△).

kinase C-independent receptor desensitization and internalization. Lai and El Fakahany [55] concluded that desensitization induced by the phorbol ester is heterologous and that protein kinase C is not involved in the desensitization induced by the m_1 muscarinic cholinergic agonists.

Receptor uncoupling from its associated G-protein or second messenger

Under prolonged stimulation by the agonist, receptors may undergo a rapid (and readily reversible) uncoupling from the G-protein that mediates the second messenger response, leading to the return of second messenger levels near basal levels in a few minutes. The mechanism of such uncoupling remains controversial but it may involve activation of protein kinases and subsequent phosphorylation of the receptor is thought to induce the uncoupling or the internalization of receptors like the β-adrenergic receptors [44–46].

All the muscarinic receptor subtypes are coupled to the two main membrane-transducing systems through a GTP-dependent mechanism [56–58]. Muscarinic receptor activation may lead to either stimulation of PI breakdown by stimulation of phospholipase C (m_1, m_3, m_5 subtypes), mainly found in neuronal tissue, or inhibition of cAMP accumulation by inhibition of adenylyl cyclase activity (m_2 or m_4 subtypes) [12, 16, 17]. Moreover, in some cells (such as neuroblastoma cells N1E-115) activation of muscarinic receptors leads to a calcium-dependent stimulation of NO-synthetase resulting in guanylate cyclase activation and increase in cGMP level [59]. The third cytoplasmic loop of the receptors plays a crucial role in the selective coupling to their intracellular effector system [37, 60–62].

Pretreatment of NG108-15 cells, which possess m_2 muscarinic receptors, with carbachol (100 μM) resulted in a rapid loss of the carbachol-induced inhibition of adenylyl cyclase that was blocked by atropine [5]. In cells bearing muscarinic receptors of the m_1 subtype (mainly N1E-115 cells), the coupling to PI hydrolysis and its desensitization has been studied in many experimental conditions [20, 52, 63–68]. Cholinergic stimulation led, within a few minutes to an increase of PI metabolism which slowly decreased and reached the level of unstimulated cells (after 20 min in N1E-115 cells), indicating desensitization. This effect was blocked by atropine [69]. In the same cells, short-term incubation of intact cells with cholinergic agonists resulted in rapid and specific desensitization of agonist-induced effects such as the transient increase in cGMP synthesis [63, 64]. This rapid desensitization was not related to changes in muscarinic receptor number studied with [^{3}H]QNB. There was good correlation between the ability of various muscarinic receptor agonists to induce a rapid decrease in muscarinic cell-surface receptors and the stimulation of cGMP synthesis in these cells. The kinetics of the rapid disappearance of [^{3}H]methylscopolamine binding sites and that of agonist-induced desensitization of receptor-mediated increase in cGMP were similar [53]. Moreover, the desensitized cGMP formation and the number of cell surface receptors recover simultaneously [70]. It is noteworthy that in these experiments, receptor internalization and desensitization did not occur very rapidly. Interestingly, recent studies in Chinese

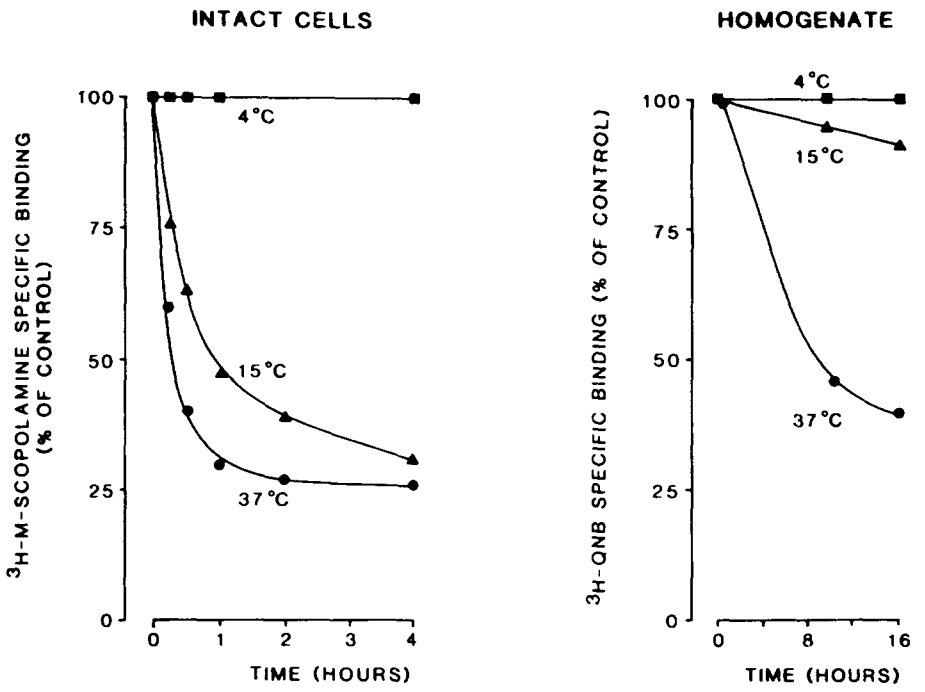

Fig. 2. Time course of carbachol-induced effect on muscarinic receptors in intact neurons and cell homogenate at different temperatures. Intact neurons were incubated with carbachol at 4° (■), 15° (▲) or 37° (●). The specific binding to muscarinic receptors was measured using [^{3}H]methylscopolamine (0.5 nM) in intact neurons and [^{3}H]QNB (1 nM) in the homogenate.

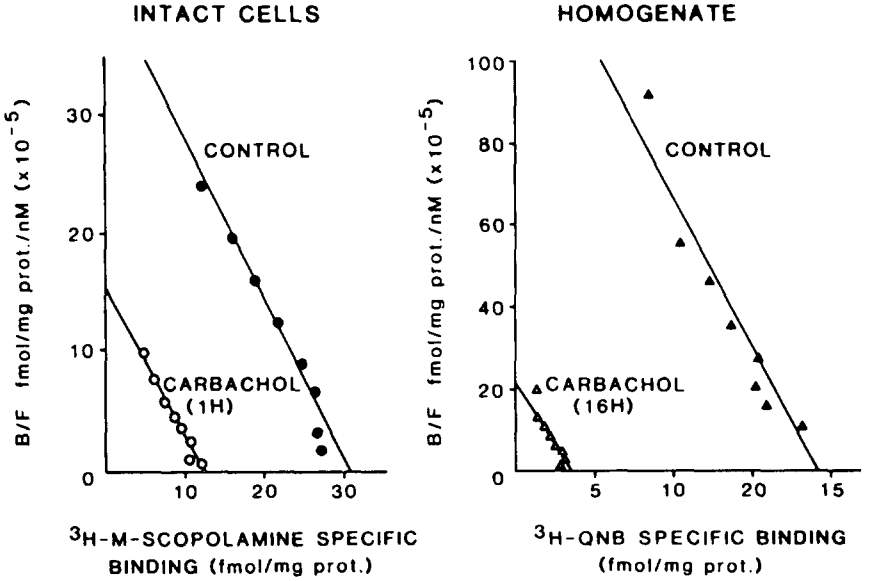

Fig. 3. Scatchard analysis of [^{3}H]methylscopolamine specific binding in intact neurons and [^{3}H]QNB specific binding in the homogenate from cells incubated in the presence or absence of carbachol. Intact neurons were incubated at 37° with or without carbachol (0.5 mM) for 1 hr and the specific binding was measured on intact cells, at 4°, with [^{3}H]methylscopolamine. When the cells were incubated for 16 hr with carbachol, the specific binding of [^{3}H]QNB was measured on the total particulate fraction from the cells (results are means of three different experiments).

hamster ovary cells, transfected with m_3 receptors, showed that rapid desensitization of the agonist-induced phosphoinositide response (inositol-1,4,5-trisphosphate formation) was not accompanied by a decrease of cell surface muscarinic receptors. The rapid desensitization and recovery of inositol-1,4,5-trisphosphate response was correlated with the transient receptor-mediated mobilization of intracellular calcium [71]. Therefore, a fast receptor desensitization might occur without receptor internalization but the role of uncoupling and/or phosphorylation in this process is still unclear.

Internalization of muscarinic receptors

Referring to the rapid agonist-induced internalization of β-adrenergic receptors, demonstrated with the hydrophilic ligand CGP 12177 [72], [^{3}H]-*N*-methylscopolamine, allowed us to study the fate of cell surface muscarinic receptors after incubation of intact cells with agonists. The incubation of cultured cells (NG108-15, N1E-115, IMR-32, PC12, neuronal cells from rat cerebral cortex or rat cerebellum) in the presence of agonists (acetylcholine, carbachol, oxotremorine or pilocarpine) resulted in the rapid decrease of the [^{3}H]-*N*-methylscopolamine specific binding to intact cells (about 80% of the control values). The reduction of [^{3}H]-*N*-methylscopolamine binding sites was dependent on the duration of the incubation and reached a steady state within 60 min (Fig. 1). The effect was concentration and temperature dependent. Experimentally, the most effective way to inhibit receptor mediated internalization is to reduce the temperature to between 0° and 4° [73–75]. The rapid disappearance of the cell surface receptors was observed when incubation with cholinergic agonist was performed at 37° and 15° but not at 4° (Fig. 2); indeed, the binding experiment with the labeled ligand was always performed at 4° (for 30 min) to avoid receptor reappearance during that phase of the experiment. The effect was rapidly reversible when the incubation of the cells was prolonged after the agonist had been washed away (Fig. 1).

The reduction of [^{3}H]-*N*-methylscopolamine binding corresponded to a decrease in receptor number at the cell surface (B_{max}) without change of receptor affinity (Fig. 3). The total amount of receptors in the homogenate of the same cells was not reduced. The agonist-induced disappearance of cell surface receptors was never complete: about 30% of the total receptor population, apparently resistant to agonist treatment, was not internalized. The explanation for this is not known but, it is probably not due to inefficent linkage to the G-proteins since G-protein coupling does not seem to be a prerequisite for receptor internalization.

Several drugs and chemical compounds, as well as changes in ionic buffer composition, were tested in order to inhibit (or potentiate) the carbachol-induced rapid disappearance of [^{3}H]-*N*-methyl-

Table 1. Effect of drugs on the agonist-induced short-term disappearance of [^{3}H]-methylscopolamine (2 nM) specific binding to cell surface muscarinic receptors of intact neuroblastoma × glioma NG108-15 cells

	[^{3}H]methylscopolamine specific binding % of control values (fmol/mg protein)
Control	100 (22.86 ± 1.09)
Carbachol (0.5 mM)	28 (6.43 ± 0.74)
Acetylcholine (1 mM with eserine)	24
Atropine (10 nM)	92
Carbachol (0.5 mM) + atropine (10 nM)	78
Colchicine (1 mM)	89
Carbachol (0.5 mM) + colchicine (1 mM)	29
Methylamine (20 mM)	101
Carbachol (0.5 mM) + methylamine (20 mM)	25

Drugs, transmitters or incubation conditions that were without influence on the [^{3}H]methylscopolamine specific binding to intact cells and on the disappearance of muscarinic receptors on cell surface induced by carbachol incubated for 1 hr with intact NG108-15 cells were dansylcadaverin (200 μM), monensine (250 μM), vincristine (0.55 μM), azide (10 mM), valinomycine (5 μM), NEM (10 μM), GppNHp (10 μM), tunicamycine (0.1 μg/mL), 3-4-diaminopyridine (1 μM), A 23187 (1 μM), ouabain (1 mM), apamine (10 nM), phencyclidine (1 μM), verapamil (10 μM), flunarizine (10 μM), noradrenaline (100 μM), serotonin (100 μM), (−)- or (+)-etomidate (10 μM), spiperone (1 μM), yohimbine (1 μM), puromycine (50 μM), cycloheximide (50 μM), met-enkephalin (10 μM) in the presence of puromycin (50 μM), morphine (1 μM), imipramine (1 μM), diphenylhydantoine (1 μM), nicotine (10 μM); 55 or 155 mM K^+ buffer, calcium free buffer (with or without 1 mM EGTA).

Intact cells were incubated for 1 hr in the presence or absence of drugs or transmitters. The [^{3}H]methylscopolamine specific binding was measured in intact cells at 0–4°. The results are expressed as a percentage of control values.

scopolamine specific binding to cell surface muscarinic receptors (Table 1). The rapid agonist-induced decrease in [^{3}H]-*N*-methylscopolamine binding sites was partially prevented in the presence of the muscarinic antagonists atropine or scopolamine, but only under experimental conditions that allowed a complete wash out of the antagonists in order to avoid their persistence on the receptor sites. Methylamine is a weak basic compound which accumulates within lysosomes [76], increases lysosomal pH [77, 78] and can impair receptor endocytosis, recycling or degradation [79, 80]. Preincubation of the cells with methylamine (1 mM) did not inhibit the carbachol-induced decrease of muscarinic receptor internalization in NG108-15 cells (Table 1). Dansylcadaverine is an amine which inhibits the uptake of most but not all ligands [79, 81, 82], probably by inhibition of transglutaminase, an enzyme capable of cross-linking proteins in coated pits. Bacitracin is also an amine that inhibits internalization of some ligands but not others [79, 83–85]. Monensin, a carboxylic ionophore for monovalent ions [86], inhibits the processing of proteins in the Golgi apparatus and the endocytosis of some ligand–receptor complexes [87, 88] and interrupts the recycling of some receptors [89–91]. Monensin, like other alkylamines, raises the pH of endosomes and lysosomes [92]. Dansylcadaverine (0.2 mM), bacitracin (10 μM) and monensin (0.25 mM) did not prevent the agonist-induced rapid disappearance of cell surface muscarinic receptors. This is compatible with the fact that lysosomes are not involved in short-term agonist-induced decrease of muscarinic receptor number. Colchicine and vincristine (poisons which interrupt the motion of vesicles along the microtubules), azide (which inhibits mitochondrial activity), antimycin A (that reduces intracellular ATP concentration) and valinomycine (an ionophore which reduces K^+ gradients) did not inhibit receptor internalization. NEM reduces the binding affinity of agonists for the muscarinic receptor (m_2 subtype) and greatly reduces the ability of agonists to inhibit the adenylyl cyclase activity [12, 34, 93, 94]. We observed a significant reduction in the affinity of agonists for the muscarinic receptor when a NG108-15 cell homogenate was incubated with NEM, but preincubation and/or incubation of the intact cells in the presence of NEM was without influence on the carbachol-induced rapid decrease in [^{3}H]-*N*-methylscopolamine specific binding. GTP (0.1 μM) and GppNHp (a stable analog of GTP) induce a shift from the high-affinity agonist binding state to the low affinity [94] but did not influence the receptor internalization rate.

When NG108-15 cells were incubated in a K^+ free buffer, the rapid decrease in [^{3}H]-*N*-methylscopolamine specific binding was less marked and this was more significant when the cells were preincubated in the K^+ free buffer for 30 min before further incubation in the presence of carbachol (Fig. 4). The effect of K^+ free buffer was reversed in the presence of rubidium. Larkin *et al.* [95] showed that the depletion of intracellular K^+ (by incubation of the intact cells in isotonic K^+ free buffer) caused a rapid reduction in the rate of endocytosis of receptor bound low-density protein (LDL) and EGF in human

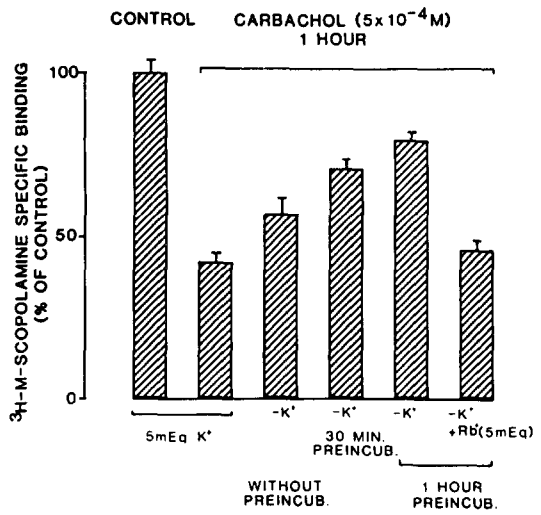

Fig. 4. Effect of depletion of intracellular K^+ (by incubation in isotonic K^+-free buffer) on the carbachol-induced decrease of muscarinic receptors in intact neuronal cells. Intact NG108-15 cells were incubated with or without carbachol for 1 hr in 5 mEq K^+ or K^+-free buffer (with or without preincubation for 30 min or 1 hr). In some experiments, rubidium (Rb, 5 mEq) was added in the K^+-free preincubation buffer of carbachol treated cells. [^{3}H]-methylscopolamine specific binding was measured in intact neurons and expressed as a percentage of the control values (experiments were performed three to six times in triplicate).

fibroblasts. Depletion in intracellular K^+ reversibly arrests coated pits formation and receptor mediated endocytosis in fibroblasts. Depletion of intracellular K^+ also induces a reduction in several anabolic processes such as inhibition of protein and DNA synthesis [96] and a loss of response to polypeptide growth factors such as EGF, vasopressin and insulin [97]. It was recently shown that EGF receptors may bind to assembly proteins (adaptins), which anchor the clathrin lattice to the inner surface of the cell membrane. Although unoccupied receptors may associate with the adaptins, binding of EGF to its receptors increased the interaction with adaptins. The depletion of K^+ blocked endocytosis of EGF receptors and increased receptor-adaptin association, suggesting that receptor adaptin interactions occur before coated pits are assembled [98]. Our results in K^+-depleted neuronal cells suggest the role of coated pits in the internalization mechanism of muscarinic receptors activated by agonists.

High K^+ concentrations (55 or 155 mM K^+) in the incubation medium for a short time (up to 60 min) were without effect on the agonist-induced decrease in [^{3}H]-*N*-methylscopolamine specific binding but we observed that prolonged (24 hr) neuronal cell depolarization in medium containing a high potassium concentration (25 mM) induced an increase in muscarinic receptors that could be due to increased receptor synthesis or, as suggested by Liles and Nathanson [99], by inhibition of muscarinic receptor degradation. However, the effect of cell depo-

larization varies from one cell type to the other, since Shaw *et al.* [100] observed a muscarinic receptor down-regulation in cortex slices after a 4-hr depolarization using veratridine or high external potassium concentration.

The rapid disappearance of muscarinic receptors was specifically induced by muscarinic agonists and was related to the potency of these agonists. Other receptor sites (opiate, α2-adrenergic) have been measured in NG108-15 cells after incubation with carbachol and were unchanged (not shown here). The reappearance of [^{3}H]methylscopolamine specific binding on intact cells was rapid, temperature dependent (Fig. 1) and was not accelerated by depolarization of the cells in high K^+ medium nor by A23187 or BAY K8644. The reappearance of muscarinic receptors at the cell surface, after short term incubation with carbachol, was not inhibited by protein synthesis inhibitors such as cycloheximide (50 μM) or puromycine (50 μM).

Muscarinic receptor down-regulation

A down-regulation of muscarinic receptors, induced by the agonists, corresponds to a decrease in the number of cellular receptors. Muscarinic agonists were found to shorten muscarinic receptor half-life [4, 26]. We observed a muscarinic receptor down-regulation after prolonged incubation of the cells in the presence of cholinergic agonists. The down-regulation was concentration and temperature dependent. There was no down-regulation when the cells were incubated at 0–4° for 16 hr with the agonist but more surprisingly, there was no down-regulation at 16° (Fig. 2). This could be explained by the absence of fusion of lysosomal and vesicular membranes at this temperature. Interestingly, there was a similar temperature dependence for desensitization of muscarinic receptor mediated cGMP formation [64].

The agonist-induced muscarinic receptor down-regulation was fully prevented by pharmacological concentrations of muscarinic antagonists (atropine or scopolamine, 50 nM). The down-regulation was also inhibited by so-called lysosomotropic compounds such as methylamine (Table 2) and was not accompanied by down-regulation of opiate or α2-adrenergic receptors. Colchicine (1–10 μM) completely inhibited the slow carbachol-induced receptor down-regulation (Table 2).

Pertussis toxin (which inactivates the inhibitory G-protein Gi involved in the adenylyl cyclase response of m_2-muscarinic receptor subtype) did not prevent the agonist-induced muscarinic receptor down-regulation [101]. Therefore, a functional Gi does not seem to be required for down-regulation of muscarinic cholinergic receptor in NG108-15 cells. Higher doses of the toxin were reported to induce a decrease in muscarinic receptors of neuronal cells that were chronically treated (5 days) suggesting a possible role of Gi in the control of muscarinic receptor turnover [102]. After down-regulation, there was a slow increase in receptor number which was inhibited, to a large extent, by puromycine or cycloheximide suggesting that receptor reappearance corresponds to newly synthesized receptors in the neuronal cells.

The signals which determine whether an internalized receptor will be degraded or recycled are not known. Antagonists, despite binding to receptors with a very high affinity, never induce receptor internalization and they are never endocytosed by a receptor-mediated process. On the contrary, endocytosis of high affinity agonists like neuropeptides or growth factors has been reported simultaneously with their receptor internalization. The persistent binding of the agonist on the receptor after internalization, might be one of the signals that leads to receptor degradation. In these conditions, the receptor might be maintained in phosphorylated or affinity conformations which would not allow receptor recycling to the cell surface. Neurotensin receptors, which are internalized with the peptide, do not rapidly recycle at the cell surface [103] but, when internalization is induced by a peptide derivative which displays a lower affinity for the receptor, this peptide is thought to dissociate and the receptor returns to the cell surface [104]. Prolonged receptor occupancy by the agonist might trigger the degradation of the internalized receptor. It has been shown that the endocytic vesicles that contain a newly ingested ligand become rapidly acidified after the internalization process [105] and this can induce the dissociation of the ligand (EGF, insulin, α2-macroglobulin) from the receptor [85, 87]. However, this is not the rule: iron transport protein transferrin remains associated with the transferrin receptor under similar acidic conditions although the bound iron is released [106, 107]. The affinity of most cholinergic agonists is low for the muscarinic receptor (micro- to millimolar range) and the dissociation rate is very fast. Moreover, there is a rapid degradation of the transmitter by acetylcholinesterase and a very efficient choline re-uptake system by the cholinergic neurons. For these reasons, it is unlikely that muscarinic agonists will always be internalized with the receptors, but this might be the case for a small proportion of the receptors, leading to their progressive degradation after several hours of incubation with the agonists.

The cytoskeleton plays a key role in the down-regulation of several receptors such as the transferrin receptor. Down-regulation of transferrin receptors can be mediated by phorbol esters and is associated with receptor phosphorylation by activated protein kinase C [108, 109]. An intact cytoskeleton is needed to allow transferrin receptor internalization since colchicine inhibited phorbol-ester receptor internalization, whereas colchicine did not inhibit receptor phosphorylation, suggesting that in this model, receptor phosphorylation is not sufficient to induce its internalization and down-regulation. An intact cytoskeleton is also required for recycling of newly synthesised receptors such as insulin receptors [110]. In heart cell culture [111] and in organ cell culture of guinea pig vas deferens [112, 113], agonist-induced down-regulation has been suggested via endocytosis and cytoskeletal dependent transport of internalized receptors to intracellular degradation sites. Higuchi *et al.* [114] also reported that the recovery of muscarinic cholinergic receptors following agonist-induced down-regulation was reduced by drugs that interfere with microtubules.

Table 2. Effect of drugs on the down-regulation of muscarinic receptors induced by 16 hr incubation of NG108-15 cells with carbachol

	[^{3}H]QNB specific binding (% of control values; fmol/mg protein)
Control	100 (43.5 ± 5.2)
Carbachol (0.5 mM)	36 (15.7 ± 2.6)
Acetylcholine (1 mM; with eserine)	33
Scopolamine (50 nM)	108
Carbachol + scopolamine (5 nM)	66
scopolamine (50 nM)	103
scopolamine (500 nM)	91
Colchicine (1 mM)	94
Carbachol + colchicine	96
Methylamine (20 mM)	91
Carbachol + methylamine	80

[^{3}H]QNB (1.5 nM) was measured in the homogenate from these cells. Intact cells were incubated for 16 hr in the presence or absence of drugs or transmitters. The [^{3}H]QNB specific binding was measured in the cell homogenate and the result expressed as a percentage of control values (experiments were performed at least three times in triplicate).

In neuronal cells in culture, nocodazole (an antimicrotubular agent dissimilar from colchicine) but not cytochalasin B (an antimicrofilament agent) was reported to reduce muscarinic receptor down-regulation and to inhibit the externalization of newly synthesised receptors. We observed that colchicine (up to 10 μM) did not inhibit muscarinic receptor internalization (Table 1) but reduced receptor down-regulation in NG108-15 (Table 2), PC12 cells and neurons from rat forebrain.

The third cytoplasmic loop of the muscarinic receptor was shown to be very important in agonist-induced internalization mechanism [115, 116]. Moreover, this third loop of the muscarinic (m_1) receptor is also involved in long-term agonist-mediated down-regulation of a number of receptors. It is not known whether the removal of the major portion of the third intracellular loop may stabilize the receptor against degradation by reducing the accessibility to intracellular proteases or is required for targeting internalized receptors to lysosomes [117].

The various agonist-induced mechanisms leading to cell desensitization clearly differ in their duration and may be different according to the type of second messenger coupled to the receptors or receptor subtypes. In most cases, several successive or simultaneous reactions that cannot be easily dissociated are involved in desensitization. The contributions of three of the main mechanisms of β-adrenergic receptor desensitization: phosphorylation by protein kinase A, by βARK [44–46] and sequestration or redistribution of β-receptors to other cell compartments such as light vesicles [72, 118, 119], were investigated by selective inhibition [120]. This led to the conclusion that the three mechanisms were distinct, but had probably overlapped physiological roles in controlling receptor function. A complete blockage of the desensitization required the concurrent inhibition of the three pathways [120]. This is probably also true in the case of muscarinic receptor desensitization.

Besides desensitization, another aspect of agonist-induced delayed effects will be increasingly studied in the future. Internalized receptors might have some physiological roles in the cells, and those which will not undergo recycling to the cell surface might initiate or participate in slow and complex intracellular regulatory processes including down-regulation, retrograde axonal transport or specific modulation of mRNA levels [121–125].

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